REMARKS

Receipt is acknowledged of the office action mailed April 4, 2005. In the April 4, 2005 office action claims 24-97 were rejected under 35 U.S.C. §112, first paragraph, for lack of enablement, and claims 60-97 were rejected under 35 U.S.C. §112, second paragraph, as being indefinite. Claims 24-26, 28, 29, 31, 32, 36-38, 44-46, 49, 51, 52, 55-57, 60-70, 73-77, 79 and 90-93 were rejected under 35 U.S.C. §102 (e) as anticipated by United States Patent No. 5,789,554 and under 35 U.S.C. §102 (b) as anticipated by WO 96/04925. Claims 60-65, 67-69 and 90-95 were rejected under 35 U.S.C. §102 (b) as anticipated by Juweid *et al.* Claims 24-26, 28, 29, 31, 32, 36-38, 44-47, 49, 51, 52, 55, 56, 60-69, 73-77 and 90-93 were rejected under 35 U.S.C. §103(a) as obvious over United States Patent No. 5,789,554 in view of Li *et al.* Claims 24-29, 31, 32, 36-38, 44-46, 49, 51, 52, 55-57, 60-70, 73-77 and 90-93 were rejected under 35 U.S.C. §103(a) as obvious over United States Patent No. 5,789,554 in view of United States Patent No. 5,106,955. Claims 24-26, 28, 29, 31, 32, 36-42, 44-46, 48, 49, 51, 52, 55-57, 60-70, 73-77 and 90-93 were rejected under 35 U.S.C. § 103(a) as obvious over United States Patent No. 5,789,554 in view of United States Patent No. 5,686,072 and PCT publication WO 95/09917. Claims 24-26, 28, 29, 31, 32, 34-39, 44-46, 48, 49, 51, 52, 55-57, 60-70, 73-77 and 90-93 were rejected under 35 U.S.C. § 103(a) as obvious over United States Patent No. 5,789,554 in view of European Patent Application No. 510949. Claims 24-38, 43-46, 49, 51, 52, and 55-97 were rejected under 35 U.S.C. § 103(a) as obvious over United States Patent No. 5,789,554 in view of United States Patent No. 5,698,178. Claims 24-38, 43-46, 49, 51, 52 and 55-97 were rejected under 35 U.S.C. § 103(a) as obvious over WO 96/04925 in view of United States Patent No. 5,698,178. Claims 60-97 were rejected under 35 U.S.C. § 103(a) as obvious over Juweid *et al.* in view of United States Patent No. 5,698,178. Claims 24-97 were provisionally rejected under the doctrine of obviousness-type double patenting over claims 24-44 of co-pending application No. 10/314,330.

Claims 24-97 were pending at the issuance of the April 4, 2005 Office Action. Claims 1-23 previously were canceled. Claims 28-35, 46, 48-51, 53, 54 and 90 are canceled herein and claims 24, 36-39, 52, 55 and 60-62 have been amended. The amendments are fully supported by the specification.

Claims 24-27, 36-45, 47, 52, 56-89 and 91-96 are pending for consideration, which is respectfully requested in view of the foregoing amendments and following remarks.

I. Formalities

A. Information Disclosure Statement

As requested by the Examiner, Applicant encloses herewith copies of references A10, A12, A17 and A19 listed in the IDS dated October 1, 2001. Applicant has also reviewed the titles and journal listings of references A12, A17 and A19 in order to correct any inaccuracies in the IDS.

Applicant has provided herewith a Form PTO/SB/08A (08-00) with the "correct" titles and journal listings. Applicant respectfully requests that the Examiner consider these references and acknowledge consideration thereof by initialization of the Form S PTO/SB/08A (08-00).

B. Specification

Priority Claim

The Examiner has objected to the specification for not listing the provisional application or the U.S. application for which the current application is a continuation-in-part.

On December 4, 2003 Applicant filed a Second Preliminary Amendment, which amended the first paragraph on page one of the specification in order to provide the complete listing of priority information. Nevertheless, Applicant has amended the specification herein to resubmit the complete listing of priority information for the instant application.

Trademarks

As requested, Applicant has reviewed the specification to assure that all trademarks are properly designated. Applicants have amended the specification herein to make any necessary corrections to the specification.

II. Rejection under § 112, first paragraph

Claims 24-97 are rejected under 35 U.S.C. § 112, first paragraph, because the specification allegedly does not enable fragments of human, humanized or chimeric anti-CD22 antibodies. Although Applicant respectfully submits that antibody fragments are well known in the art, Applicant has adopted the Examiner's suggestion to add specific language to the claims in order to better define the claimed invention. Specifically, Applicant has amended independent claims 24 and 60 to recite that an antibody fragment is selected from the group consisting of $F(ab')_2$, Fab', Fab and scFv. Applicant submits that these amendments obviate the rejection.

III. Rejection under § 112, second paragraph

Claims 60-97 are rejected under 35 U.S.C. § 112, second paragraph, because the specification allegedly does not clarify the term "indirectly" as it relates to the attachment of the agent to the antibody. Applicant believes that the claims fully comply with § 112, second paragraph, because they clearly define how the agent is attached to the antibody. However, in order to expedite the prosecution of this application Applicant has amended claim 60, 61 and 62 to state that a linkage is used to connect the therapeutic agent to the anti-CD22 antibody.

Applicant further points out that with respect to claims 85 and 86 the specific type of linkage is recited. For example, claim 85 reads: "the therapeutic agent is attached indirectly to the anti-CD22 antibody or antibody fragment by means of an aminodextran, a polypeptide carrier or a chelating agent that is attached to the anti-CD22 antibody or antibody fragment . . ." and claim 86 reads: "the therapeutic agent is attached indirectly to an anti-CD22 antibody fragment via a carbohydrate moiety introduced into the light chain variable region of the antibody fragment" (emphasis added).

For the above-stated reasons, Applicant respectfully requests withdrawal of the entire rejection.

IV. Rejections under 35 U.S.C. § 102

A. *United States Patent No. 5,789,554, WO 96/04925, and Juweid et al.*

Claims 24-26, 28, 29, 31, 32, 36-38, 44-46, 49, 51, 52, 55-57, 60-70, 73-77, 79 and 90-93 are rejected under 35 U.S.C. § 102(b) and (e) as anticipated by WO 96/04925 and

United States Patent No. 5,789,554, respectively. Claims 60-65, 67-69 and 90-95 are rejected under 35 U.S.C. §102 (b) as anticipated by Juweid *et al.*

Applicant has amended independent claims 24 and 60 to more particularly describe the claimed invention. Specifically, amended claim 24 is directed to immunoconjugates that are comprised of (i) at least one human, humanized or chimeric anti-CD22 antibody or a fragment thereof, wherein the antibody fragment is selected from the group consisting of F(ab')2, Fab', Fab and scFv and (ii) a drug or a radioisotope, wherein said radioisotope is other than ^{131}I, wherein the immunoconjugate is used in combination with a naked anti-CD20 mab.

None of the cited references disclose or teach the claimed immunoconjugate and/or the use of the claimed immunoconjugate in combination with a naked anti-CD20 mab.

Amended claim 60 is directed to immunoconjugates that are comprised of (i) at least one human, humanized or chimeric anti-CD22 antibody or a fragment thereof, wherein the antibody fragment is selected from the group consisting of $F(ab')_2$, Fab,' Fab and scFv (ii) a therapeutic agent selected from the group consisting of a drug-and a radioisotope, wherein the therapeutic agent is attached indirectly via a linkage to the anti-CD22 antibody or antibody fragment or is attached directly to the anti-CD22 antibody or antibody fragment-via a free sulfhydryl group.

None of cited references describe an immunoconjugate where the therapeutic agent is attached indirectly via a linkage to the anti-CD22 antibody or antibody fragment or is attached directly to the anti-CD22 antibody or antibody fragment-via a free sulfhydryl group.

In order to reject a claim under 35 USC § 102, the Examiner must demonstrate that each and every claim limitation is contained in a single prior art reference. *See Scripps Clinic & Research Foundation v. Genentech, Inc.*, 18 USPQ2d 1001, 1010 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 90 (Fed. Cir. 1986); *see also* MPEP § 2131 (August 2001). None of the cited references describe each and every element of the instantly claimed invention and therefore applicants respectfully request that the rejection under 35 USC § 102 be withdrawn.

V. Rejections under 35 U.S.C. § 103

Claims 24-26, 28, 29, 31, 32, 36-38, 44-47, 49, 51, 52, 55, 56, 60-69, 73-77 and 90-93 are rejected under 35 U.S.C. §103(a) as obvious over United States Patent No. 5,789,554 in view of Li *et al.* Claims 24-29, 31, 32, 36-38, 44-46, 49, 51, 52, 55-57, 60-70, 73-77 and 90-93 are rejected under 35 U.S.C. §103(a) as obvious over United States Patent No. 5,789,554 in view of United States Patent No. 5,106,955. Claims 24-26, 28, 29, 31, 32, 36-42, 44-46, 48, 49, 51, 52, 55-57, 60-70, 73-77 and 90-93 are rejected under 35 U.S.C. § 103(a) as obvious over United States Patent No. 5,789,554 in view of United States Patent No. 5,686,072 and PCT publication WO 95/09917. Claims 24-26, 28, 29, 31, 32, 34-39, 44-46, 48, 49, 51, 52, 55-57, 60-70, 73-77 and 90-93 are rejected under 35 U.S.C. § 103(a) as obvious over United States Patent No. 5,789,554 in view of European Patent Application No. 510949. Claims 24-38, 43-46, 49, 51, 52, and 55-97 are rejected under 35 U.S.C. § 103(a) as obvious over United States Patent No. 5,789,554 in view of United States Patent No. 5,698,178. Claims 24-38, 43-46, 49, 51, 52 and 55-97 are rejected under 35 U.S.C. § 103(a) as obvious over WO 96/04925 in view of United States Patent No. 5,698,178. Claims 60-97 are rejected under 35 U.S.C. § 103(a) as obvious over Juweid *et al.* in view of United States Patent No. 5,698,178.

In an effort to advance prosecution of the instant application, Applicant has amended independent claims 24 and 60 to more particularly describe the claimed invention. To the extent that the Examiner seeks to apply the rejection to the amended claims, Applicant respectfully traverses.

All claims are presumed initially to be non-obvious. A *prima facie* case of obviousness requires three elements: (1) a teaching or suggestion of all of the claim limitations; (2) a suggestion or motivation to modify or combine the teachings of the applied prior art; and (3) a reasonable expectation of success in reaching the claimed invention. The Examiner bears the initial burden of supporting any *prima facie* assertion of obviousness with adequate facts. MPEP § 2142 (Feb. 2000).

Here, none of the cited references, either alone or in combination, disclose all the elements of the amended claims. Accordingly, the first element of a *prima facie* case of obviousness cannot been satisfied and withdrawal of the rejection respectfully is requested.

VI. Rejections for Obviousness-Type Double Patenting

Claims 24-97 are rejected under the doctrine of obviousness-type double patenting over claims 24-44 of co-pending application No. 10/314,330. Applicant respectfully requests that this rejection be held in abeyance until the indication of allowable subject matter in the instant application, at which time Applicant will consider the filing of a suitable terminal disclaimer.

CONCLUSION

Applicant respectfully asserts that the amendments presented above should be entered as they place this case in condition for allowance by clarifying the invention and responding the Examiner's suggestions or concerns. In view of the amendment and remarks, Applicants respectfully requests that all objections and rejections be withdrawn and that a notice of allowance be forthcoming. The Examiner is invited to contact the undersigned attorney for Applicant at (202) 912-2197 for any reason related to the advancement of this case.

Respectfully submitted,

Date: October 4, 2005

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of 1

Complete If Known	
Application Number	09/965,796
Filing Date	October 1, 2001
First Named Inventor	David M. Goldenberg
Group Art Unit	1642
Examiner Name	HARRIS, Alana M
Attorney Docket Number	40923-0079 US3

OTHER PRIOR ART – NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	A10	Oliver W. Press; "Prospects for the Management of Non-Hodgkin's Lymphomas with Monoclonal Antibodies and Immunoconjugates"; The Cancer Journal from Scientific American, vol. 4, Supplement 2, Pgs. S19-S26	
	A12	M. Ghetie et al., "Evaluation of Ricin A Chain-containing Immunotoxins Directed against CD19 and CD22 Antigens on Normal and Malignant Human B-Cells as Potential Reagents for in Vivo Therapy," Cancer Research, 1988, 48, 2610-2617	
	A17	J. Leonard et al., "Epratuzumab, A New Anti-CD22, Humanized, Monoclonal Antibody for the Therapy of Non-Hodgkin's Lymphoma (NHL): Phase I/II Trial Results," Blood, vol 94, no. 10 suppl. 1 part 1, 1999, Abstract #404	
	A19	W. M. J. Vuist et al., "Potentiation by Interleukin 2 of Burkitt's Lymphoma Therapy with Anti-Pan B (Anti-CD19) Monoclonal Antibodies in a Mouse Xenotransplantation Model," Cancer Research, July 15, 1989, 49, 3783-3788	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

A10

ORIGINAL ARTICLE

Prospects for the Management of Non-Hodgkin's Lymphomas with Monoclonal Antibodies and Immunoconjugates

Oliver W. Press, MD, PhD, *Seattle, Washington*

■ PURPOSE

To review the role of monoclonal antibody constructs in the treatment of B-cell malignancies.

■ PATIENTS AND METHODS

The efficacy and tolerability of unmodified monoclonal antibodies, immunotoxins, and radioimmunoconjugates have been investigated in patients with hematologic B-cell malignancies. Response rates, durability of responses, and tolerability were the principal measures of treatment outcome.

■ RESULTS

Investigators from several institutions have documented response rates ranging from 25% to 95% in lymphoma patients suffering relapses who were treated with antibody constructs targeting the CD19, CD20, CD22, DR, or idiotypic immunoglobulin epitopes on malignant B-cell lymphomas. Chimeric anti-CD20 antibodies and ^{131}I-labeled anti-CD20 antibodies appear particularly promising, producing response rates of 50% to 95%. Complete remissions (CRs) appear to be more frequent and durable with radiolabeled anti-CD20 antibodies (33% to 85% CR rate) than with unmodified chimeric anti-CD20 antibodies (6% to 10% CR rate), although a randomized comparison has not yet been made.

■ CONCLUSION

Monoclonal antibodies provide promising new reagents for the treatment of patients with B-cell non-Hodgkin's lymphomas. Impressive response rates have been achieved in clinical trials using unmodified monoclonal antibodies, immunotoxins, and radioimmunoconjugates, although the durability of responses is still under scrutiny. Durability may be improved when the antibodies are used in conjunction with chemotherapy or stem cell transplantation. *(Cancer J Sci Am 1998;4:S19-S26)*

Key words: Monoclonal antibodies, immunotoxins, radioimmunoconjugates, B-cell non-Hodgkin's lymphomas

Over the past 15 years, several research groups have investigated the role of monoclonal antibodies in the treatment of hematologic malignancies. Theoretically, the binding of unmodified murine monoclonal antibodies to tumor-associated antigens on the surface of malignant lymphoid cells, followed by activation of the host immune system, leads to tumor eradication by several mechanisms, including complement-mediated cytolysis; antibody-dependent cellular cytotoxicity; release of cytokines, such as tumor necrosis factor or interleukin-1; interruption of anti-idiotypic networks; and induction of apoptosis. Initial experiments conducted in the 1980s on mouse models of leukemias and lymphomas indicated that dramatic cures could often be obtained with the use of unmodified murine monoclonal antibodies. For example, in one notable trial, 100% of mice were cured of T-cell lymphoma by injection of a tumor-specific anti-Thy1.1 antibody, whereas all untreated animals died within 3 to 4 weeks.[1] Experiments such as these generated considerable enthusiasm for initiating clinical studies aimed at curing human hematologic malignancies. Unfortunately, the results in clinical trials to date have been less dramatic than those obtained in animal models, although in recent studies clinical responses have been more encouraging.[2-14]

■ EFFICACY AND TOLERABILITY OF MONOCLONAL ANTIBODIES IN B-CELL LYMPHOMAS

Unmodified Mouse Monoclonal Antibodies

B-cell lymphomas have been selected by many researchers as the ideal tumor model for clinical trials of monoclonal antibodies because they express a variety of well-defined cell surface antigens for which high-quality

From the Division of Medical Oncology, University of Washington Medical Center, Seattle, Washington.

The author has received or will receive benefits for personal or professional use from a commercial party related directly or indirectly to the subject of the article.

Dr. Press received the anti-B1 antibody (Coulter Pharmaceuticals, Palo Alto, CA), the MB-1 antibody (Idex Pharmaceuticals, La Jolla, CA), and the 1F5 antibody (Oncogen Inc., Seattle, WA) free of charge for these studies. In addition, Coulter Pharmaceuticals provided a part-time data manager for one of these studies.

Supported by grants from the National Institutes of Health (P01 CA44991 and R01CA56696).

Reprint requests: Oliver W. Press, MD, PhD, Box 356043, Division of Medical Oncology, University of Washington Medical Center, Seattle, WA 98195-6043.

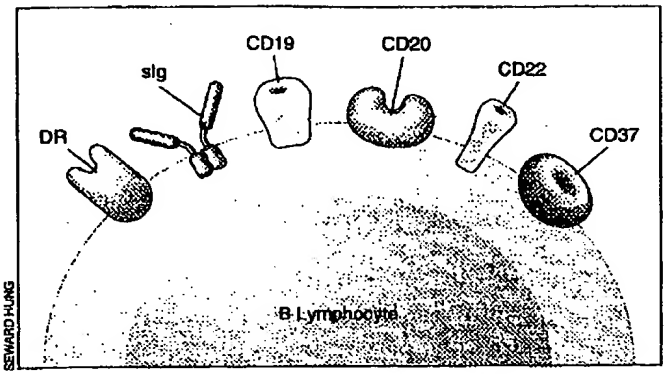

Figure 1 Antigenic targets on B cells include the class II human leukocyte antigen (DR), surface idiotypic immunoglobulin (sIg), CD19, CD20, CD22, and CD37.

monoclonal antibodies have been prepared (e.g., surface idiotypic immunoglobulin, CD19, CD20, CD21, CD22, CD37, and class II human leukocyte antigen DR [Fig.1]). The results of some of the most prominent unmodified monoclonal antibody trials conducted to date are summarized in Table 1.[8,10,15-22]

The most successful clinical trials using tumor-specific anti-idiotypic antibodies were conducted by Levy and co-workers at Stanford University.[15-17] In three sequential trials, anti-idiotypic antibodies, alone or in combination with interferon alfa or chlorambucil, produced objective responses in 57% to 73% of treated patients. Although the median response duration was only 6 to 7 months in these trials, patients who had complete responses often achieved remissions lasting longer than 5 years. Despite the therapeutic success and the theoretical importance of these seminal studies, however, the logistic and financial constraints of preparing tumor-specific anti-idiotypic antibodies have precluded widespread adoption of this approach or its commercialization.

Most investigators have subsequently selected pan-B antibodies that recognize B-lymphocyte differentiation antigens for trials involving patients with B-cell lymphomas. Many of these groups, including our own, have focused on the CD20 antigen as the target molecule (Fig. 2). The CD20 antigen is an unglycosylated phosphoprotein that traverses the cell membrane four times and has both its amino and carboxy termini inside the cell. The CD20 antigen does not circulate in the bloodstream and, therefore, does not act as a "blocking factor" or otherwise impede tumor cell targeting. Furthermore, CD20–anti-CD20 antigen-antibody complexes are not internalized by the cell[23,24]—thereby allowing cell surface-bound antibody to persist for protracted lengths of time, which permits optimal interaction between the antibody and host effector cells or complement.

In the mid-1980s, we initiated clinical trials using the unmodified murine anti-CD20 monoclonal antibody 1F5, and we demonstrated that it was capable of causing rapid clearance of circulating tumor cells from the peripheral blood (PB) of four treated patients.[18] In addition, transient shrinkage of lymph nodes was observed in some patients, and 90% regression of malignant lymph nodes was observed in the patient treated with the highest dose of antibody. Unfortunately, these responses were not durable, and pathologic lymphocytosis and lymphadenopathy recurred within 2 to 6 weeks. Consequently, we and others began to explore methods of enhancing the magnitude and duration of responses that are achievable with murine monoclonal antibodies.

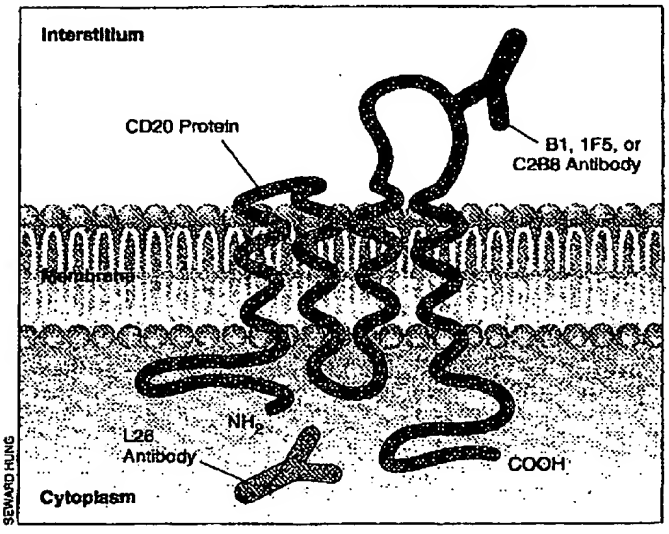

Figure 2 CD20 is a B-lymphocyte–specific, nonglycosylated, "four-pass" surface membrane phosphoprotein encoded on chromosome 11, which has both its carboxy and amino termini inside the cell. It is believed to function as a calcium channel and is involved in B-cell activation and progression through the cell cycle. The B1, 1F5, and C2B8 antibodies bind to the extracellular domain of CD20, whereas the L26 antibody binds to an intracellular epitope. Reproduced in modified form with permission.[40]

Chimeric Human-Mouse Anti-CD20 Antibodies

One approach that has been successfully pursued entails molecular engineering of a human-mouse chimeric anti-CD20 antibody (C2B8; Rituxan™ [rituximab]; IDEC Pharmaceuticals, San Diego, CA; Genentech, San Francisco, CA), which contains murine immunoglobulin variable regions grafted onto human IgG1 kappa domains.[8-10,25] In vitro experiments have convincingly demonstrated that human Fc regions markedly enhanced the interaction between the chimeric antibody and human effector cells and complement, compared with the parent murine antibody (2B8). This dramatically enhanced the ability of the chimeric C2B8 antibody to initiate complement-mediated lympholysis and antibody-mediated cellular cytotoxicity in vivo, compared with 2B8.[25] Furthermore, the chimeric C2B8 antibody was significantly less immunogenic than 2B8 in primates and humans.

Table 1. Summary of Clinical Responses in B-Cell Lymphoma Patients Treated with Unmodified Murine, Chimeric, or Humanized Antibodies

Study	Disease	Antibody (Antigen)	Antibody Type	Evaluable Patients	Clinical Responses
Maloney[8]	B-NHL	C2B8 (CD20)	Chimeric	34	3 CR 14 PR
McLaughlin[10]	B-NHL	C2B8 (CD20)	Chimeric	151	9 CR 67 PR
Levy[16-17]	B-NHL PLL	Anti-idiotype	Murine	15	2 CR 7 PR 2 MR
Press[18]	B-NHL	1F5 (CD20)	Murine	4	1 PR 1 MR
Dyer[19]	B-NHL CLL ALL	Campath 1M (CD52) Campath 1G (CD52)	Rat Rat	18	18 MR
Hale[20]	B-NHL	Campath 1H (CD52)	Humanized	2	1 CR 1PR
Hekman[21]	B-NHL	LLB (CD19)	Murine	6	1 PR 1 MR
Hu[22]	B-NHL	LYM-1 (DR)	Murine	10	3 MR

Abbreviations: B-NHL, B-cell non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia; PLL, prolymphocytic leukemia; CR, complete remission; PR, partial remission; MR, minor response.

As might be expected, the chimeric C2B8 antibody has demonstrated significantly improved clinical efficacy compared with murine anti-CD20 antibodies.[8-10] In a preliminary single-center trial, 17 of 34 patients (50%) with relapses of low-grade non-Hodgkin's lymphoma (NHL) treated intravenously with 375 mg/m^2 of C2B8 antibody weekly for 4 consecutive weeks achieved objective responses, including 3 complete remissions (CRs; 9%) and 14 partial remissions (PRs; 41%).[8] Toxicity was minimal and generally limited to the peri-infusional period. Side effects, which consisted of mild and transient fevers, chills, rashes, and hypotension, occurred more commonly during the first infusion of C2B8 and became much less common during subsequent treatments, presumably because of the protracted depletion of circulating B lymphocytes induced by the first infusion. No long-term adverse effects have been observed, and myelosuppression has generally been clinically insignificant. No human anti-mouse antibody (HAMA) or human anti-chimeric antibody (HACA) responses were observed in this trial.[8] The chimeric C2B8 antibody can be administered in an outpatient setting, and the treatment is completed in 22 days.

A subsequent multicenter trial confirmed the efficacy of C2B8 in patients with clinically chemoresistant low-grade or follicular lymphoma.[10] In this trial, 76 of 151 patients (50%) achieved remissions. Unfortunately, only 6% to 10% of the remissions were complete, and the median response duration was only 11 months. Of interest, 18 of 23 patients (78%) who were treated with C2B8 while in relapse after autologous bone marrow transplantation (ABMT) responded. No HACA responses were observed. Molecular analysis with the polymerase chain reaction (PCR) assay revealed that in response to C2B8 therapy, a fraction of patients who initially had t(14;18) translocation-positive lymphoma cells in their bone marrow (BM) and PB became PCR negative (i.e., achieved a molecular CR) in those compartments (approximately 68% in the PB and 50% in the BM).[26] The significance of this observation is unclear, however, because many of the patients with an apparent molecular CR in their BM and PB had persistent palpable pathologic lymphadenopathy. It is possible that antibody therapy effectively eradicates disease in the easily accessible PB and BM compartments without achieving similar clearance of malignant cells from large lymph nodes or tumor masses, which are more difficult for antibody molecules to permeate. Nevertheless, despite this unresolved issue, the convincing efficacy of the C2B8 antibody and its modest toxicity have led the United States Food and Drug Administration to approve C2B8 for the treatment of NHL. Current clinical studies are evaluating methods for improving CR rates and the durability of responses by combining C2B8 with conventional chemotherapy (cyclophosphamide, doxorubicin, vincristine, prednisone [CHOP]) or other biologic agents (e.g., interferon alfa) or by employing it as adjuvant therapy after BM or stem cell transplantation.

Immunotoxins

An alternative approach to augmenting the efficacy of monoclonal antibodies involves conjugating the anti-

body to cytotoxic plant or bacterial toxins to produce an "immunotoxin."[27] For immunotoxins to be effective, the antibody to which the toxin is conjugated must be internalized by the cell, because toxins such as diphtheria toxin and ricin A chain kill cells by irreversibly inhibiting elongation factor 2 or ribosomes, respectively, which are located in the cytosol. The most successful antibodies used for synthesizing immunotoxins target either CD19 (B4, HD37) or CD22 (RFB4) on B-cell lymphomas or CD3, CD5, or CD7 on T-cell malignancies. A series of clinical trials have been conducted by Vitteta and collaborators in Texas and by Grossbard and Nadler and coworkers in Boston using anti–B-cell immunotoxins.[28-33] In patients with advanced, relapsed, and refractory B-cell lymphomas treated with immunotoxins, response rates varied from 11% to 30% (Table 2).[27-33] The dose-limiting toxicities associated with immunotoxins included vascular leak syndrome, hepatotoxicity, and myalgias.

Radiolabeled Antibodies

Another mechanism for augmenting the tumoricidal activity of monoclonal antibodies consists of conjugating them to radionuclides such as iodine-131 (^{131}I), yttrium-90 (^{90}Y), and copper-67 (^{67}Cu) to form "radioimmunoconjugates," which appear to possess several advantages over other antibody constructs. First, they do not rely on recruitment of host immune effector mechanisms to kill tumor cells; this is important because the immune system is often suppressed or defective in cancer patients. Furthermore, the beta particles emitted by the decay of ^{131}I or ^{90}Y are capable of killing cells from a distance of several cell diameters, thereby permitting radioactive "cross-fire" from antigen-positive cells that can kill neighboring antigen-negative tumor cell variants.[34] Antigen-negative tumor cells would elude destruction by unmodified antibodies or immunotoxins. Moreover, radioactive cross-fire mitigates the deleterious consequences of heterogeneous antibody distribution within large tumor masses, which often results in suboptimal concentrations of antibody at the center of tumors or at locations distant from blood vessels. A variety of radiolabeled antibodies have been evaluated in clinical trials (Table 3).[2,3,5-7,11,14,35-38]

Lym1 antibody. Preliminary studies with the ^{131}I-labeled Lym1 antibody were conducted at the University of California, Davis, by DeNardo and colleagues. The Lym1 antibody targets an aberrant class II HLA molecule. In patients with advanced lymphomas, treatment with the ^{131}I-labeled Lym1 antibody yielded approximately a 50% response rate.[14]

Anti-CD20 antibodies. Recently, Kaminski and colleagues at the University of Michigan demonstrated that nonmyeloablative doses of ^{131}I-labeled anti-CD20 antibody (anti-B1, Bexxar®; Coulter Pharmaceuticals, Palo Alto, CA) produce durable CRs in patients with relapses of B-cell lymphoma.[6] In a phase I/II trial, 34 patients with low-grade, intermediate-grade, and high-grade lymphomas were treated with ^{131}I-labeled anti-B1 antibody according to a dose escalation schema (Fig. 3). Patients were initially administered nonradiolabeled (i.e., cold) anti-B1 antibody at a dose of 0, 135, or 685 mg, followed by dosimetric infusion of trace ^{131}I-labeled anti-B1 antibody (15 to 20 mg; 5 to 10

Table 2. Summary of Clinical Responses in B-Cell Lymphoma Patients Treated with Immunotoxins

Study	Disease	Antibody (Antigen)	Toxin	Evaluable Patients	Clinical Responses
Foss	CTCL NHL Hodgkin's	IL-2 (IL-2R)	DAB389	73	6 CR 10 PR
Vitetta[28]	B-NHL	RFB4-Fab' (CD22)	dg-RTA	14	5 PR
Amlot[29]	B-NHL	RFB4-IgG (CD22)	dg-RTA	24	1 CR 5 PR
Sausville[30]	B-NHL	RFB4-IgG (CD22)	dg-RTA	16	4 PR 3 MR
Stone[31]	B-NHL	HD37-IgG (CD19)	dg-RTA	32	1 CR 2 PR
Grossbard[32]	B-NHL ALL CLL	B4 (CD19)	blocked-ricin	25	1 CR 2 PR 8 MR
Grossbard[33]	B-NHL ALL CLL	B4 (CD19)	blocked-ricin	34	2 CR 3 PR 11 MR

Abbreviations: CTCL, cutaneous T-cell lymphoma; B-NHL, B-cell non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia; CR, complete remission; PR, partial remission; MR, minor response; DAB389, truncated form of diphtheria toxin; dg-RTA, deglycosylated ricin A-chain; IL-2, interleukin-2; IL2R, interleukin-2 receptor.

mCi). The rationale for infusing cold anti-B1 antibody was to partly saturate the readily accessible antigenic sites in the bloodstream and spleen. In this way, the subsequently infused ^{131}I-labeled anti-B1 antibody is better able to penetrate lymph nodes and large tumor masses, which are less accessible to antibodies. Sequential biodistribution studies demonstrated that preinfusion of cold anti-B1 antibody (650 mg) resulted in delivery of higher doses of radiation to tumor sites, less radiation to normal organs, and a more favorable antibody biodistribution. On the basis of the calculated pharmacokinetics of the ^{131}I-labeled antibody, a therapeutic infusion of ^{131}I-labeled anti-B1 antibody was then delivered to patients in a dose escalation manner according to the conjugation of an amount of ^{131}I to the antibody that was calculated to deliver 25 to 85 cGy whole-body irradiation. This approach produced objective responses in all 13 patients with low-grade lymphoma, and 10 of those patients (77%) achieved a CR. In six patients, the duration of CRs ranged from 16+ to 31+ months. Hematologic toxicity was dose limiting at 75 cGy (whole body dose). In a recent phase II study conducted by Kaminsky and colleagues in 10 newly diagnosed low-grade lymphoma patients, all 10 patients achieved objective responses to ^{131}I-labeled anti-B1 antibody.[39] The first three patients treated have achieved probable CRs, and the remaining patients show signs of continued regression.

Knox and colleagues at Stanford University have conducted a similar trial using ^{90}Y-labeled anti-CD20 antibodies to treat relapses in B-cell lymphoma patients.[7] Four patients were treated with ^{90}Y-labeled anti-B1 antibody, and 14 patients received ^{90}Y-labeled C2B8. These patients received a 1 to 2.5 mg/kg preinfusion of cold anti-CD20, followed by 13 to 50 mCi of ^{90}Y-labeled anti-CD20. Six patients (33%) achieved CRs, and seven patients (39%) achieved PRs, with a median response duration of 6 months.

Other antibodies. A number of clinical trials have also explored the effects of a variety of other radioimmunoconjugates (Table 3). Royston and colleagues treated nine patients with ^{90}Y-labeled anti-idiotypic antibodies, and obtained two CRs (22%) and one PR (11%).[38] Juweid and colleagues treated 24 chemorefractory patients with ^{131}I-labeled anti-CD22 antibody, LL2, or F(ab')$_2$ fragments of LL2.[37] In this trial, 21 patients received nonmyeloablative doses ranging from 15 to 343 mCi (15 to 50 mCi per cycle, for a maximum of seven cycles), and three patients received myeloablative doses (90 mCi/m^2) followed by ABMT. Seventeen of 21 patients and two of three patients were assessable in the nonmyeloablative and myeloablative groups, respectively. In the nonmyeloablative treatment group, a 29% response rate was reported (one CR, two PRs, and two minor responses [MRs]). In addition, one patient achieved a CR after the imaging dose of ^{131}I-labeled anti-CD22. In the ABMT group, two patients achieved PRs lasting 3 and 8 months, respectively.

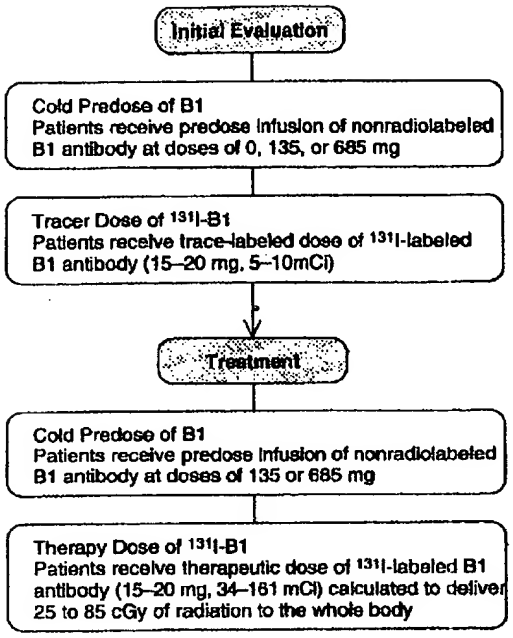

Figure 3 Treatment approach for B-cell lymphoma patients using the ^{131}I-labeled anti-CD20 antibody (anti-B1, Bexxar®; Coulter Pharmaceuticals, Palo Alto, CA).

■ EFFICACY AND TOLERABILITY OF MONOCLONAL ANTIBODIES IN CONJUNCTION WITH STEM CELL TRANSPLANTATION

High-Dose Radioimmunotherapy as a Single Agent with Autologous Stem Cell Transplantation

Our group in Seattle decided to test the maximum potential of radioimmunoconjugates for the treatment of B-cell lymphomas by administering myeloablative doses of ^{131}I-labeled anti–B-cell antibodies in conjunction with purged autologous BM or PB stem cell support. In a phase I dose escalation trial, we studied the biodistribution of ^{131}I-labeled anti–B-cell antibodies in 43 patients by administering sequential weekly infusions of 0.5, 2.5, and 10 mg/kg of anti-CD20 or anti-CD37 antibodies trace-labeled with 5 to 10 mCi of ^{131}I.[2] Twenty-four of 43 patients (56%) had favorable antibody biodistributions, as defined by the absorption of higher radiation doses by all tumor sites than by critical normal organs. Anti-CD20 antibodies, anti-B1 and 1F5, were superior to anti-CD37 antibodies at targeting malignant tissue, and an optimal protein dose of 2.5 mg/kg was established for the anti-B1 antibody. However, large tumor burdens (> 500 cc) and splenomegaly adversely affected the delivery of ^{131}I-labeled anti–B-cell antibodies to tumor sites. Nineteen patients received therapeutic infusions of ^{131}I-labeled anti–B-cell antibodies (12 patients received anti-B1, one patient received 1F5, and six patients received anti-CD37 antibody MB-1) calculated to deliver a specified maximum dose of radiation to critical normal organs, varying from 10 to 31 Gy, in an escalat-

Table 3. Summary of Clinical Responses in B-Cell NHL Patients Treated with Radioimmunoconjugates

Author	Disease	Antibody (Antigen)	Isotope	Evaluable Patients	Clinical Responses
Press[2]	B-NHL	MB1 (CD37) or B1 (CD20)	^{131}I	19	16 CR 2 PR 1 MR
Press[3]	B-NHL	B1 (CD20)	^{131}I	21	17 CR[a] 1 PR 1 MR
Kaminski[5,6]	B-NHL	B1 (CD20)	^{131}I	28	14 CR 8 PR
Knox[7]	B-NHL	B1, B28 (CD20)	^{90}Y	18	6 CR 7 PR
Goldenberg[11]	B-NHL	LL2 (CD22)	^{131}I	5	2 PR 2 MR
DeNardo[14]	B-NHL	LYM-1 (HLA-DR)	^{131}I ^{67}Cu	51	12 CR 16 PR
Kaminski[35]	B-NHL	MB-1 (CD37)	^{131}I	9	1 CR 2 PR 3 MR
Czuczman[36]	B-NHL	OKB7 (CD21)	^{131}I	18	1 PR 12 MR
Juweid[37]	B-NHL CLL	LL2 (CD22)	^{131}I	17	1 CR 2 PR 2 MR
Royston[38]	B-NHL	Anti-Idiotype	^{90}Y	9	2 CR 1 PR

[a]One patient initially reported as having a PR subsequently achieved a CR.
Abbreviations: B-NHL, B-cell non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; CR, complete remission; PR, partial remission; MR, minor response.

ing-dose manner. Eighteen patients (95%) achieved objective responses, including 16 CRs and two PRs. Currently, eight patients remain in continuous CR 46 to 95 months after therapy. Nonhematologic toxicities, generally mild at doses < 23 Gy, included nausea, fever, and elevated thyroid-stimulating hormone levels. At lung doses > 27 Gy, two of four patients (50%) developed reversible cardiopulmonary toxicity, thus establishing the maximum tolerated dose to the lungs at approximately 27 Gy.

In a subsequent phase II trial, we treated 21 multiply relapsed NHL patients with 2.5 mg/kg of anti-B1 antibody that was labeled with an amount of radioiodine (345 to 785 mCi) calculated to deliver 25 to 27 Gy to dose-limiting critical normal organs.[3] Of the 21 patients who received therapeutic infusions, 17 (81%) eventually achieved CRs, one achieved a PR, and one achieved an MR. A high-grade transformed immunoblastic large-cell lymphoma progressed in one patient during therapy, and the patient died 1.5 months later. The median response duration in this trial was 38 months. Kaplan-Meier estimates of 6-year overall and progression-free survival are 68% and 42%, respectively, and 82% and 57%, respectively, for patients with indolent lymphomas (Fig. 4). Eighty-three percent of patients experienced longer remissions after ^{131}I-labeled anti-B1 antibody therapy than with prior chemotherapy.

As expected, all patients treated with myeloablative doses of ^{131}I-labeled anti-CD20 antibodies developed severe BM aplasia and required BM (19 patients) or PB stem cell (2 patients) transplantation. Neutropenia and fever developed in 70% of patients, and approximately one third of patients developed infections, one of which proved fatal (polymicrobial sepsis). Transient mild elevations in transaminase were noted in approximately one third of patients, but no cases of significant veno-occlusive liver disease were noted. Vomiting occurred in approximately 30% of patients, but other common transplant-associated toxicities, such as alopecia and mucositis, were rare (< 5% of patients). One commonly observed delayed toxicity was subclinical hypothyroidism, which developed in 60% of patients 6 to 12 months after therapy and was manifested by asymptomatic elevations in levels of thyroid-stimulating hormone. None of the patients in the phase I/II studies have developed myelodysplasia or acute leukemia after a cumulative follow-up of 107 person-years. Two patients have developed solid tumors (superficial transitional cell carcinoma of the bladder and colon carcinoma), each approximately 3 years after ^{131}I-labeled anti-B1 antibody therapy. In

27% of patients, HAMA responses developed, usually between 3 and 12 months after treatment.

Radioimmunotherapy in Conjunction with High-Dose Chemotherapy and Stem Cell Transplantation

Despite these encouraging results, there is considerable room for improvement, particularly for patients with intermediate-grade and high-grade lymphomas, for whom the median response duration was only 24 months after use of single-agent ^{131}I-labeled anti-B1 antibody. In an effort to improve the durability of remission, we initiated a trial to investigate the combination of ^{131}I-labeled anti-B1 antibody with high-dose chemotherapy and stem cell transplantation. This phase I/II dose escalation trial is designed to evaluate the safety and efficacy of ^{131}I-labeled anti-B1 antibody (at doses delivering 21 to 27 Gy to critical normal organs) in combination with high-dose VP-16 (0 to 60 mg/kg) and cyclophosphamide (100 mg/kg) with autologous stem cell support. Thirty-five patients have been treated since the trial began in 1995, and approximately 80% of patients are currently in disease-free remission after 1 to 34 months' follow-up (unpublished results). However, the toxicities associated with this combination chemoradioimmunotherapy regimen are considerably greater than those associated with single-agent ^{131}I-labeled anti-B1 antibody therapy; they include mucositis, alopecia, nausea, reversible veno-occlusive liver disease (one patient), and disseminated varicella-zoster virus infection (one patient). Larger patient populations, longer follow-up, and additional randomized trials will be needed to determine whether this combined modality approach will improve remission durations compared with single-agent ^{131}I-labeled anti-B1 antibody therapy.

CONCLUSION

Impressive response rates have been documented by investigators using anti–B-cell monoclonal antibodies, immunotoxins, and radioimmunoconjugates in patients with relapses of B-cell lymphoma. Objective remissions have been achieved in 50% to 95% of treated patients in studies that targeted the CD20 antigen with chimeric or radiolabeled antibodies. Although these results are exciting, most patients will ultimately suffer relapses and die from their lymphomas, indicating that further innovations are needed. It appears likely that therapeutic antibodies will provide maximum clinical benefit when administered to patients with minimal tumor burden in conjunction with high-dose chemotherapy and stem cell support.

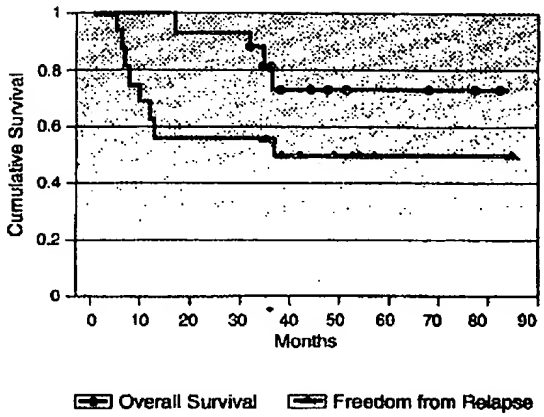

Figure 4 Kaplan-Meier estimates of relapse-free and overall survival of all 29 patients with relapses of B-cell lymphomas treated in Seattle, Washington, in phase I and II trials with ^{131}I-labeled anti-B1 antibody as a single agent followed by autologous bone marrow or stem cell transplantation (O. Press, unpublished results).

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[CANCER RESEARCH 48, 2610-2617, 1988]

Evaluation of Ricin A Chain-containing Immunotoxins Directed against CD19 and CD22 Antigens on Normal and Malignant Human B-Cells as Potential Reagents for *in Vivo* Therapy[1]

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ABSTRACT

Ricin A chain-containing immunotoxins (IT-As) specific for the human B-cell antigens, CD22 and CD19, were constructed using the monoclonal antibodies, HD6 and HD37, respectively. IT-As were prepared by coupling intact antibodies, $F(ab')_2$, or Fab' fragments to native or chemically deglycosylated ricin A chain. The IT-As were then evaluated for cytotoxicity to normal and neoplastic human B-cells *in vitro* with the major objective of appraising their suitability for *in vivo* therapy of human B-cell tumors. The IT-As prepared with both the HD6 and HD37 antibodies were specifically toxic to normal B-cells and to most of the neoplastic B-cell lines tested. However, the IT-As prepared from HD6 were generally more potent than those prepared from HD37. On Daudi cells, to which the two antibodies bound in similar numbers and with similar affinities, IT-As prepared with intact HD6 antibody or its Fab' fragment were 10-fold and 1.5- to 4-fold more potent, respectively, than the corresponding HD37 IT-As. The IT-As constructed from intact HD6 antibody and native or deglycosylated A chain reduced protein synthesis in Daudi cells by 50% at a concentration of 1.2×10^{-11} M indicating that they were only 5-fold less toxic to the cells than ricin itself. Intact HD37 IT-As produced equivalent inhibition of protein synthesis at 1.5×10^{-10} M. With both antibodies, IT-As constructed from the Fab' fragments were 10- to 20-fold less potent than their intact antibody counterparts. Different neoplastic B-cell lines varied in sensitivity to the IT-As. In most cases, their sensitivity correlated with the levels of CD19 and CD22 antigens expressed. Neither HD6 nor HD37 IT-As affected the ability of normal human bone marrow cells to form granulocyte-macrophage colony-forming units in soft agar, suggesting that both antigens are absent from these progenitor cells. Examination of sections of frozen human tissues using immunoperoxidase staining procedures indicated that the antibodies did not bind to a panel of normal tissues lacking B-lymphocytes. These results suggest that HD6 and HD37 IT-As are candidates for *in vivo* therapy in humans with certain B-cell tumors. However, HD6 IT-As are more potent, reduce protein synthesis more completely, and hence appear to be the ITs of choice for treating tumors expressing the CD22 antigen.

INTRODUCTION

Novel antitumor agents have been prepared in several laboratories by coupling the A chain of the plant toxin, ricin, to monoclonal antibodies directed against tumor-associated antigens (reviewed in Refs. 1 and 2). These IT-As[3] specifically kill malignant cells *in vitro*. However, *in vivo* studies in rodents and, more recently, in humans have met with mixed success. In rodents, good antitumor effects have generally been obtained in leukemia models whereas solid tumors appear less responsive (1, 2). In humans, the antitumor effects of IT-As in a small number of leukemia patients have been modest and transient (3) and, in a Phase I trial in 22 melanoma patients, only one patient had a complete remission and three had partial remissions (4).

Recent investigations of the fate of IT-As in rodents have revealed that several factors reduce their efficacy *in vivo*. (*a*) The carbohydrates on the A chain are recognized by the parenchymal and nonparenchymal cells of the liver and induce the rapid clearance of the IT-A from the bloodstream (5–7). This can be prevented by dgA (8). (*b*) The large size of IT-As prepared from intact antibodies (M_r 180,000) reduces their access to neoplastic cells in the spleens of leukemic mice.[4,5] IT-As formed using the Fab' fragment of antibody are smaller (M_r 80,000) and home to the neoplastic cells in larger amounts.[4] (*c*) Free antibody, which is generally a contaminant of IT-A preparations, is much longer lived than the IT-A *in vivo* and so can mask the tumor antigens from IT-A that is subsequently administered (9). This can be overcome by removing the free antibody from the IT preparation by chromatography on blue sepharose (10). (*d*) IT-As containing intact antibodies are unstable *in vivo* because the disulfide bonds generated by cross-linking agents such as SPDP or 2-iminothiolane are prone to reduction (5). This can be substantially overcome either by using a new cross-linking agent, SMPT (11), which introduces a hindered disulfide bond with greater stability, or by using Fab'-As (12, and Footnote 5), which contain a cystine linkage. In studies reported elsewhere, we have found that IgG-As and Fab'-As incorporating the above improvements have markedly increased therapeutic activity in mice with B-cell leukemia[5] or T-cell lymphoma (9).

We plan to conduct our first clinical trial of IT-As in patients with B-cell tumors (*e.g.*, B-lymphoma and B-cell chronic lymphocytic leukemia). The antibodies considered for use as ITs in these trials are anti-CD19 (HD37) and anti-CD22 (HD6). Both antibodies recognize B cell-restricted antigens that are expressed on a high proportion of normal and malignant B-cells (13–20). In a previous report, we demonstrated that both antibodies make effective IT-As (21). In the present study, we compared ITs containing IgG, $F(ab')_2$ or Fab' fragments of antibody coupled by different cross-linking agents (SMPT, SPDP, or Ellman's reagent, to native or dgA chain. The results

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[3] The abbreviations used are: IT-As, ricin A chain-containing immunotoxins; CFU-GM, granulocyte-macrophage colony-forming units; dgA, deglycosylated ricin A chain; DTNB, (5,5'-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent); DTT, dithiothreitol; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; ITs, immunotoxins; PBE, 0.1 M phosphate buffer with 0.003 M Na_2 EDTA, pH 7.5; PBS, phosphate-buffered saline; SMPT, 4-succinimidyl-oxycarbonyl-α-methyl-α(2-pyridyldithio)toluene; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IC_{50}, 50% inhibitory concentration.

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of these *in vitro* preclinical comparisons are the subject of this report.

MATERIALS AND METHODS

Cells. The cell lines used in these studies are described in Table 1. The human Burkitt's lymphoma line, Daudi, was the standard test cell for the majority of the studies (22). Human peripheral blood B-lymphocytes were obtained from healthy adult volunteers as previously described (21). Bone marrow was aspirated from the posterior iliac crest of healthy donors. Mononuclear cells from bone marrow samples were obtained by centrifugation over sodium metrizoate/Ficoll gradients as previously described (22).

Antibodies. The purified myeloma protein, MOPC-21 (IgG1κ), was purchased from Litton Bionetics (Charleston, SC) or was prepared from ascitic fluid of $BCL_1 \times X63$ hybridoma cells secreting both IgMλ and IgG1κ (MOPC-21) (23). The hybridoma cell lines HD6 (anti-CD22) (24) and HD37 (anti-CD19) (24) were kindly provided by Drs. Dorken and Moldenhauer, Heidelberg, Germany. Purified HD6 and HD37 antibodies (murine IgG1κ) were prepared from ascitic fluid or culture medium by ammonium sulfate precipitation (final concentration of 45%). The precipitates were dissolved and dialyzed against the appropriate buffer and further purified by chromatography on hydroxylapatite (25) (BioRad, Biogel, HT) at pH 7.2 (phosphate buffer), SP-Sephadex (26) (Pharmacia, Piscataway, NJ) at pH 4.0 (citrate buffer) or *Staphylococcal* Protein A-Sepharose (27) (Pharmacia) at pH 9.0 (borate buffer).

The purity of the IgG1 preparations was tested by SDS-PAGE. The IgG1 isolated from ascites was also tested by double diffusion and immunoelectrophoresis using antisera to mouse serum (Litton Bionetics). The preparations were >90% pure but contained traces of an unidentified M_r 100,000 protein.

Preparation of $F(ab')_2$ and Fab' Fragments of Antibody. The IgG1 preparations were treated with pepsin (4500 units/ml) (Sigma, St. Louis, MO) for 6 h at 37°C under the following conditions: pH 3.7 (0.1 M citrate buffer); protein concentration, 2–3 mg/ml; enzyme/protein ratio, 2/100 by weight (28, 29). The digestion was terminated by raising the pH to 8.0 with 1 N NaOH. HD37 $F(ab')_2$ fragments were isolated by gel filtration on Sephacryl S-200HR (Pharmacia) equilibrated in phosphate-buffered 0.3 M NaCl. HD6 $F(ab')_2$ fragments were isolated by adsorbing the nonneutralized digest to a column (10 x 2 cm) of SP-Sephadex equilibrated in 0.1 M citrate buffer, pH 3.7, and eluting the $F(ab')_2$ fragment with PBS, pH 7.2. The yields of $F(ab')_2$ fragments ranged from 35 to 50%.

The Fab' fragment was obtained by reducing the $F(ab')_2$ fragment (5–10 mg/ml) with DTT at a final concentration of 5 mM in PBE for 1 h at room temperature. The excess DTT was removed by gel filtration on Sephadex G-25 and the thiol groups of the Fab' fragment (5 mg/ml) were derivatized with DTNB at a final concentration of 2 mM (12). The nonreacted DTNB was removed by gel filtration on a column (30 x 2 cm) of Sephadex G-25 equilibrated in PBE.

The purity of the $F(ab')_2$ and Fab' fragments was determined by SDS-PAGE and by double diffusion and immunoelectrophoresis using anti-mouse IgG sera which reacted with both Fab' and Fc fragments. The preparations were free of both Fc fragments and intact IgG.

Ricin A Chain. The A subunit of ricin was prepared as described (30) and was purchased from Inland Biologicals, Austin, TX. dgA was prepared as described by Thorpe *et al.* (8). The 50% lethal doses of native and dgA chain in 25-g mice were 0.7 mg and 0.3 mg, respectively. The IC_{50} in a cell-free rabbit reticulocyte assay was 10^{-11}–10^{-12} M for both dgA and native A chain (Ref. 30 and Footnote 5).

For conjugation with the antibody, the A chain was reduced with 5 mM DTT as described previously (12). In some cases, a small amount of radioiodinated A chain (approximately 10^6 cpm) was added to the unlabeled A chain before reduction.

Radiolabeling of Proteins. IgG1, $F(ab')_2$ fragments, Fab' fragments, and A chain were labeled with ^{125}I using the IODO-GEN reagent (Pierce, Rockville, IL) (31). The specific activities of the proteins were approximately 1 μCi/μg.

Preparation of ITs with SMPT. The preparation of IgG-A conjugates with SMPT was performed as recently described by Thorpe *et al.* (11). Briefly, SMPT dissolved in dimethylformamide was added to a solution of antibody (6.8 mg/ml) in borate buffer, pH 9, to give a final concentration of 0.11 mM. After incubation for 1 h at room temperature, the solution was filtered through a column of Sephadex G-25 equilibrated in phosphate buffer, pH 7.5, containing 1 mM Na_2 EDTA. The derivatized protein, which eluted in the void volume of the column, contained 1.5 to 2.0 α-methyl-α(2-pyridyldithio)toluoyl groups. The derivatized protein was then mixed with freshly reduced A chain (using 0.5 mg A chain per mg antibody), concentrated by ultrafiltration to about 1.5 mg total protein per ml, and stored for 3 days at 25°C under nitrogen. The mixture was then treated with 0.2 mM cysteine for 6 h at room temperature to inactivate any activated disulfide groups remaining in the IT. The IT was purified according to Knowles and Thorpe (10).

Preparation of IT-As with SPDP. IT-As were prepared using IgG or $F(ab')_2$ fragments of HD6, HD37, and MOPC-21 as previously described (12). Briefly, SPDP dissolved in dimethylformamide was added to a solution of IgG or $F(ab')_2$ (10 mg/ml) in PBE, pH 7.5, to give a final concentration of 1 mM. After 30 min at room temperature, the solution was filtered on a column of Sephadex G-25 (30 x 2 cm) equilibrated with PBE. The degree of substitution of derivatized IgG and $F(ab')_2$ fragment was 3–4 molecules pyridyldithiopropionate/molecule of protein. The derivatized protein was then mixed with reduced A chain (dissolved in PBE) using 1.3 mg A chain/mg IgG or $F(ab')_2$, concentrated by ultrafiltration to 2–3 mg/ml, and maintained for 2 h at 25°C and overnight at 4°C. The mixture was then purified (10).

Preparation of Fab'-As with DTNB. The preparation of mouse Fab'-A with DTNB-derivatized Fab' was performed as described for rabbit Fab'-A (12). Briefly, the Ellman's derivatized Fab' fragment containing 1–2 thionitrobenzoic acid-substituted thiol groups (see preparation of Fab') dissolved in PBE (5 mg/ml) was mixed at room temperature with reduced A chain using 1.3 mg A chain/mg Fab' at a final concentration of 2 mg protein/ml. The reaction between thionitrobenzoic acid-Fab' and A chain was followed by the increase of absorbance at 412 nm and was complete after 2 h at 25°C. The mixture was then immediately purified.

Purification of the IT-As. The IT-As prepared with antibody fragments were purified by affinity chromatography on Blue Sepharose using a modified version of the method described by Knowles and Thorpe (10). Chromatography was carried out in 0.05 M phosphate buffer, pH 7.0, and A chain and IT-As were eluted with 1 M NaCl prepared in the same buffer. The eluate was concentrated by ultrafiltration to 5 mg/ml and applied to Sephacryl S-200HR equilibrated with phosphate-buffered 0.3 M NaCl, pH 7.2. The peak(s) containing the purified IT-A was collected, concentrated by ultrafiltration to at least 0.5 mg/ml, and stored in aliquots at −70°C.

Molar Ratios of A Chain/Antibody. The molar ratios of A chain to antibody were calculated from the specific radioactivity of ^{125}I-A chain and the following absorption coefficients (A, 0.1%/1 cm at 280 nm): A chain, 0.77; IgG1, 1.4; $F(ab')_2$ and Fab' fragments, 1.2. With Fab'-As, molar ratios of A chain to antibody fragments of 0.85 ± 0.17 (mean and SD of 5 experiments) were obtained. With $F(ab')_2$-As, the molar ratios of A chain to antibody fragment were 2.2 ± 0.5 (mean and SD

Table 1 *Antibodies and cells used in this study*

Cell line		Immunofluorescence Staining			
		HD6 (anti-CD22)		HD37 (anti-CD19)	
		% positive	MFI[a]	% positive	MFI
Daudi	Burkitt's lymphoma	80	1673	87	1432
NAMALWA	Burkitt's lymphoma	20	222	86	378
Raji	Burkitt's lymphoma	85	1400	85	1498
ARH-77	Plasma cell line (secreting IgG)	61	426	83	586
NALM-6	Pre-B acute lymphocytic leukemia	72	495	88	2221
Jurkat	T-cell leukemia	0		0	
B cells	Normal peripheral blood	49	322	49	431

[a] MFI, mean fluorescence intensity; maximum value of 10,000.

of 4 experiments). With IgG-As, the molar ratios of A chain to antibody were 1.3 ± 0.2 (mean and SD of 6 experiments).

Binding of ITs to Daudi Cells. Binding of antibodies and IT-As to Daudi cells was evaluated by an indirect immunofluorescence assay. 10^6 Cells/0.1 ml were treated with various amounts of antibody or IT-A (2–1000 ng/0.1 ml) for 30 min at 4°C. After washing with PBS containing 0.1% sodium azide, the cells were treated with FITC-labeled goat anti-mouse immunoglobulin for 15 min at 4°C (3 μg FITC-antibody/50 μl/10^6 cells). The cells were washed and analyzed on a FACS (Becton-Dickinson, Oxnard, CA). The concentration (M) of HD6 or HD37 antibody (or the corresponding IT-A) which gave 50% of the maximum fluorescence (32) was determined.

Binding of Radiolabeled IgG and Its Fragments to Daudi Cells. Daudi cells were treated with various concentrations of radiolabeled ligands (0.02–10 μg/ml/10^7 cells) in RPMI Medium 1640 containing 10% FCS and 0.1% sodium azide and incubated for 3 h on ice. The cells were then separated from the medium by centrifugation through a mixture of dibutylphthalate and bis-ethylhexylphthalate (1.1:1.0, v/v) (33). The supernatants were discarded and the tips of the tubes containing the cell pellets were cut off and the radioactivity was determined in a gamma counter. The amount of radiolabeled ligand specifically bound was calculated by substracting the radioactivity bound in the presence of an excess of unlabeled ligand from the total radioactivity bound. In all cases, 95% or more of the binding of the radiolabeled ligand could be inhibited by the addition of a 100-fold molar excess of unlabeled ligand showing that the vast majority of binding of the radiolabeled ligand was antigen mediated. The affinity constant (K) and the number of ligand molecules bound per cell (n) under conditions of equilibration were calculated by using the Scatchard form of the equilibrium equation (34).

SDS-PAGE. Proteins were analyzed under both reducing and non-reducing conditions by SDS-PAGE on 10% gels according to Laemmli (35). Protein bands were visualized by staining the gel with Coomassie blue. The following proteins were used as standards for the estimation of molecular weight (BioRad, Richmond, CA): ovalbumin, 45 kilodaltons; bovine serum albumin, 66 kilodaltons; phosphorylase B, 92.5 kilodaltons; β-galactosidase, 116 kilodaltons; and IgG1, 150 kilodaltons.

Cytotoxicity Assay. 10^3 Cells/20 μl in RPMI Medium 1640 containing 10% FCS, glutamine, and antibiotics were distributed into triplicate wells (96-well microtiter plates) containing 100 μl of medium and concentrations of IT ranging from 10^{-12} to 10^{-7} M and incubated for 24–48 h at 37°C. The cells were centrifuged and washed twice in leucine-free RPMI 1640 containing 10% FCS and were resuspended in 200 μl of the same medium. Cells were pulsed for 4 h at 37°C in 5% CO_2 with 5 μCi [^{3}H]leucine (Amersham, Arlington, VA). Wells were harvested on a Titertek cell harvester (Flow Labs, Rockville, MD) and the radioactivity on the filters was counted in a liquid scintillation spectrometer. The percentage of reduction in [^{3}H]leucine incorporation, as compared with untreated controls, was used as the assessment of killing (23). Nine wells of untreated cells were included in each experimental group.

In some cases, the cells were treated with variable amounts of ITs for 1 h at 4 or 37°C, washed twice to remove excess IT, and further incubated for 24 h at 37°C. Cells were pulsed with radiolabeled leucine as described above.

Cytotoxicity of IT-As on Bone Marrow Cells. Human bone marrow mononuclear cells were treated with medium or with IT-As for 1 h at 37°C. The *in vitro* growth of CFU-GM was determined by soft agar cloning as described previously (22).

Incubation of IT-As with Human Plasma. Fab'-A, F(ab')$_2$-A, and IgG-A were incubated at 50 μg/ml with undiluted fresh human plasma overnight at 37°C and then used in the cytotoxicity assay in parallel with the freshly thawed IT.

Reactivity of HD6 and HD37 with Normal Human Tissues. The following tissues were tested for their reactivity with HD6 and HD37 antibodies: adrenal, bladder, brain, breast, heart, kidney, nerve, esophagus, pancreas, parathyroid, prostate, skin, testis, and thyroid. The method used was modified from the standard immunoperoxidase staining technique (36). Cryostat sections of tonsils were cut and allowed to dry onto slides overnight at room temperature in a dry atmosphere. Sections were then fixed by immersion in acetone for 10 min followed by absolute ethanol for 10 min. Fifty μl of HD6 or HD37 at appropriate dilutions in PBS containing 0.2% bovine serum albumin and 0.2% azide were added to the sections and incubated at room temperature for 1 h in a "wet box." Slides were washed in PBS for 5 min and a second layer of peroxidase-labeled rabbit anti-mouse immunoglobulin (Dako, Ltd., Bucks, UK) in 50 μl of a 1:50 dilution in PBS containing 20% normal human serum was added. Sections were incubated for 45 min at room temperature and then washed for 5 min in PBS, fixed in buffered formol-saline for 5 min, washed in tap water, and stained in 3-3'-diaminobenzidine tetrahydrochloride at 0.6 mg/ml in PBS plus 0.01% H_2O_2 for 10 min. Sections were washed briefly in tap water and counterstained sequentially in 4% iron alum solution and haematoxylin. Permanent slide mounts were prepared by dehydrating samples through alcohol and xylene and mounting them in DPX or Styrolite.

RESULTS

Characterization of Antibody Preparations and Their Binding to Several Human Cell Lines. The monoclonal murine antibodies (HD6 and HD37) both belong to the IgG1 (κ) subclass and are directed against the B-cell markers CD19 (HD37) and CD22 (HD6) (15, 19). The reactivity of these antibodies with a variety of neoplastic B-cell lines, and with normal peripheral blood lymphocytes, was determined by treating the cells with the antibodies and measuring the amount of antibody which bound with a secondary FITC-labeled goat anti-mouse immunoglobulin reagent using the FACS. The results of the FACS analyses are presented in Table 1.

Fig. 1 shows a representative Scatchard analysis of the binding of HD6 antibody and its fragments to Daudi cells. The results of this and other such analyses are summarized in Table 2 and show that intact HD6 antibodies and its F(ab')$_2$ fragments have similar affinities and bind to Daudi cells in numbers

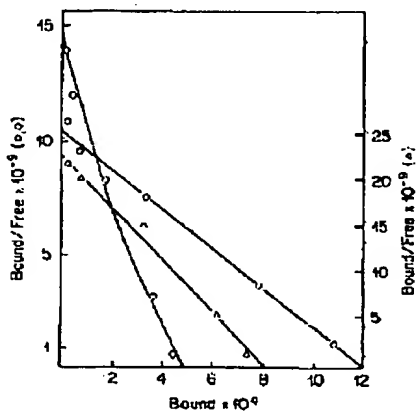

Fig. 1. Scatchard analysis of the binding of HD6 antibody and its fragments to Daudi cells. ◇, IgG antibody ($K = 2 \times 10^9$ M^{-1}, $n = 5 \times 10^4$); △, F(ab')$_2$ ($K = 1.8 \times 10^9$ M^{-1}, $n = 8 \times 10^4$); ○, Fab' ($K = 0.5 \times 10^9$ M^{-1}, $n = 12 \times 10^4$).

Table 2 *Binding parameters of HD6 and HD37 antibodies to Daudi cells*
The IgG1 and its fragments were labeled with ^{125}I. Mean of three experiments.

	Antibody			
	HD6		HD37	
Ligand	Affinity constant ($K \times 10^9$ M^{-1})	No. of molecules bound ($n \times 10^4$)	Affinity constant ($K \times 10^9$ M^{-1})	No. of molecules bound ($n \times 10^4$)
IgG1	4.2 ± 1.4	7.0 ± 1.3	3.6 ± 0.4	8.5 ± 1.4
F(ab')$_2$	3.9 ± 1.8	6.5 ± 1.0	1.7 ± 0.3	7.1 ± 1.2
Fab'	0.4 ± 0.1	10.9 ± 2.3	0.5 ± 0.1	13.0 ± 1.3

similar to those of intact HD37 antibody and its $F(ab')_2$ fragment, respectively. As expected from the results of previous studies, intact HD6 and HD37 antibodies had approximately 10-fold higher affinities than their Fab' fragments. This is probably due to the fact that the intact antibodies and their divalent $F(ab')_2$ fragments each bind to two antigens on the cell surface, whereas the monovalent Fab' fragments bind to one antigen and can therefore detach from the cell surface more readily. In support of this, the numbers of molecules of Fab' fragments that bound cells under saturating conditions exceeded the numbers of molecules of intact antibodies or $F(ab')_2$ fragments that bound by 1.6- to 1.8-fold.

Preparation and Characterization of IT-As. IT-As were constructed by coupling intact antibodies, $F(ab')_2$, and Fab' fragments to either native or dgA chain. Three monoclonal IgG1κ immunoglobulins were used: HD6, HD37, and MOPC-21 (control).

The IT-As containing IgG and $F(ab')_2$ fragments were prepared using the heterobifunctional linkers, SMPT and SPDP, respectively. Analysis of the IT-As containing IgG by SDS-PAGE (Fig. 2) indicated that they contained a single major component comprising one molecule of IgG and one molecule of A chain. Other minor bands on the gels corresponded in molecular weight to one molecule of IgG coupled to two or three molecules of A chain. The $F(ab')_2$-As were more heterogenous and contained one molecule of $F(ab')_2$ coupled to 1–3 molecule of A chain. The IT-As prepared from the Fab' fragments comprised almost exclusively one molecule of Fab' and one molecule of A chain.

Table 3 summarizes the yields of IT-A preparations, the ribosome-inactivating capacity of the A chain (following reduction of the IT-A), and the cell-binding activity of bivalent *versus* monovalent ITs. The yield of IT-As prepared using intact antibodies or their Fab' fragments was similar (19–22% with respect to the antibody component) whereas the yield of $F(ab')_2$ fragments was somewhat lower (10% with respect to the antibody component). The A chains in both divalent and monovalent IT-As retained their ability to inactivate ribosomes.

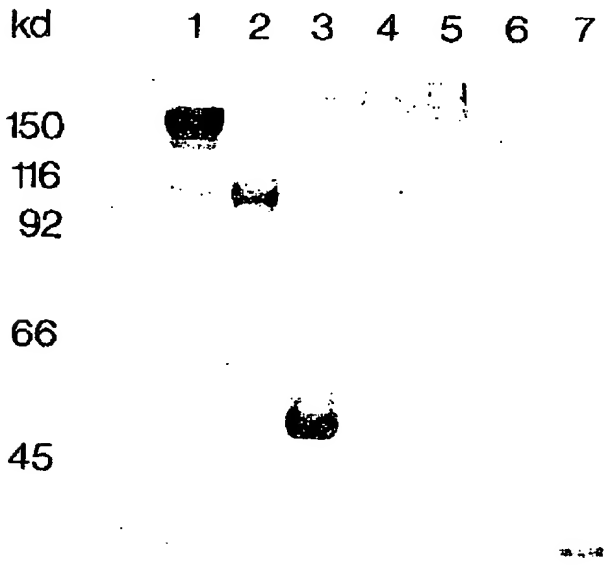

Fig. 2. SDS-PAGE analysis of IT-As and their corresponding antibodies and fragments. *Lane 1*, IgG; *Lane 2*, $F(ab')_2$; *Lane 3*, Fab'; *Lane 4*, IgG-A; *Lane 5*, $F(ab')_2$-A; *Lane 6*, Fab'-A; *Lane 7*, A chain.

Table 3 *Characterization of IT-As prepared from HD6, HD37, and dgA chain*

Immunotoxins	Yield of preparation (%)	Inhibition of cell-free protein synthesis [IC_{50} (M)][a]		Concentration giving 50% maximal fluorescence (nM)	
		HD6	HD37	HD6	HD37
IgG-dgA	22 ± 3	2.6×10^{-11}	2.9×10^{-12}	10[b]	ND[c]
$F(ab')_2$-dgA	10 ± 5	ND	ND	30	38[d]
Fab'-dgA	19 ± 4	7.8×10^{-11}	6.5×10^{-11}	56[e]	77

[a] IC_{50} for native A chain, 7.1×10^{-12}.
[b] Unconjugated IgG, 10.
[c] ND, not determined.
[d] Unconjugated $F(ab')_2$ HD6, 17, and $F(ab')_2$ HD37, 15.
[e] Unconjugated Fab' HD6, 30.

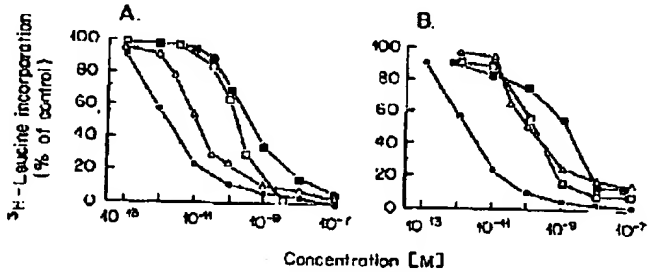

Fig. 3. Inhibition of protein synthesis by HD6- and HD37-derived ITs. (MOPC-21-A had an IC_{50} of $>10^{-7}$ M). Daudi cells were incubated for 24 h at 37°C with different concentrations of IT-As (10^{-13}–10^{-7} M), then were washed twice and pulsed for 4 h at 37°C in 5% CO_2 with 5 μCi[^{3}H]leucine. *A*, HD6: △, IgG-dgA; □, $F(ab')_2$-dgA; ■, Fab'-A; ●, ricin. *B*, HD37: △, IgG-dgA; □, $F(ab')_2$-A; ■, Fab'-A; ●, ricin.

The cell-binding ability of the $F(ab')_2$-A and Fab'-A (Table 3) was about half that of the unconjugated fragments, whereas the binding ability of the IgG-HD6-A *versus* HD6 was unchanged. The lower affinity of the $F(ab')_2$-A and Fab'-A for cells expressing the specific cell marker can be explained either by steric hindrance by the A chain or by a loss of antibody affinity due to the chemical and physicochemical procedures used to prepare the IT-A. This is in accord with the findings of Ramakrishnan and Houston (37), who reported that the $F(ab')_2$ fragment of anti-Thy-1.1 antibody coupled to pokeweed antiviral protein had a 10-fold lower affinity than the unconjugated $F(ab')_2$ fragment, whereas the affinity of an intact anti-Thy-1.1 IT was the same as that of the native antibody.

Specific Cytotoxicity of Different IT-As on Daudi Cells. The toxicity of different IT-As on Daudi cells was assessed by incubating the cells with increasing concentrations of ITs at 37°C for either 24 or 48 h. A representative experiment is shown in Fig. 3 and the results of several experiments are summarized in Table 4.

From the results summarized in Table 4, the following conclusions can be drawn. (*a*) The cytotoxic activity of IT-As constructed with HD6 (anti-CD22) was generally higher than that of IT-As prepared with HD37 (anti-CD19). With IgG-As, the difference in cytotoxic potency was about 10-fold and with Fab'-As it was 1.5- to 4-fold. HD37-A prepared with intact antibody was unable to reduce protein synthesis in Daudi cells by more than 85% at saturating concentrations (10^{-9} M or greater) whereas HD6-A at these concentrations completely inhibited protein synthesis (Fig. 3). (*b*) The toxicity of the HD6-A prepared with intact antibody was only about 5-fold less than that of ricin. (*c*) The toxic effects of the HD6-A and HD37-A were specific since IT-As prepared from intact MOPC-21 antibody, which does not bind to Daudi cells, were up to 10,000 times less toxic. (*d*) The cytotoxic activities of HD6-A and

Table 4 *Cytotoxic activity of HD6 and HD37 IT-As on Daudi cells*

IT-A[a]	Time of incubation of HD6 [IC_{50} (M)]		Time of incubation of HD37 [IC_{50} (M)]		Time of incubation of MOPC-21 [IC_{50} (M)]	
	24 h	48 h	24 h	48 h	24 h	48 h
IgG-A	$3.7 \pm 1.2 \times 10^{-11}$ (4)[b]	$1.2 \pm 0.1 \times 10^{-11}$ (4)	$1.8 \pm 0.2 \times 10^{-10}$ (4)	$1.5 \pm 0.2 \times 10^{-10}$ (4)	2.2×10^{-7} (2)	3.0×10^{-8} (2)
IgG-dgA	$1.4 \pm 0.4 \times 10^{-11}$ (4)	$1.5 \pm 0.1 \times 10^{-11}$ (4)	$1.5 \pm 0.4 \times 10^{-10}$ (4)	1.3×10^{-10} (2)	2.8×10^{-7} (2)	3.0×10^{-8} (2)
$F(ab')_2$-A	ND[c]	ND	$2.1 \pm 0.2 \times 10^{-10}$ (5)	ND	ND	ND
$F(ab')_2$-dgA	$2.7 \pm 1.3 \times 10^{-10}$ (4)	ND	ND	ND	ND	ND
Fab'-A	$9.9 \pm 5.3 \times 10^{-10}$ (4)	$4.2 \pm 0.8 \times 10^{-10}$ (6)	$2.1 \pm 0.7 \times 10^{-9}$ (4)	$3.5 \pm 0.3 \times 10^{-10}$ (4)	7.0×10^{-7} (1)	ND
Fab'-dgA	$1.0 \pm 0.3 \times 10^{-9}$ (6)	$5.2 \pm 0.8 \times 10^{-10}$ (4)	4.0×10^{-9} (2)	$3.6 \pm 0.5 \times 10^{-10}$ (4)	1.3×10^{-7} (2)	2.0×10^{-7} (2)

[a] IC_{50} for ricin toxin, $2.5 \pm 1.0 \times 10^{-12}$ (3).
[b] Numbers in parentheses, number of experiments.
[c] ND, not determined.

HD37-A constructed with divalent antibody were approximately 10 times higher than the corresponding Fab'-As. This is consistent with the results reported by Masuho *et al.* (38), Raso and Griffin (39), and Fulton *et al.* (12). (*e*) For HD6, the cytotoxic activity of IgG-A was higher than that of $F(ab')_2$-A despite the fact that both conjugates are divalent, but was in keeping with the finding that the $F(ab')_2$-A had lower binding affinity than the IgG-A. (*f*) The incubation of cells with IT-As for 48 h resulted in lower IC_{50} values (higher activity) than incubation for 24 h. Although not shown, but as reported previously (21), incubation at 4°C, followed by 24 h at 37°C, gave slightly higher IC_{50}s (lower activity). (*g*) The cytotoxicity of ITs constructed with either native or dgA chain was similar *in vitro*.

Cytotoxicity of IT-As on Other Human Leukemia and Lymphoma Cell Lines. The sensitivity of different B-cell leukemia and lymphoma cell lines to the IT-As generally correlated with their levels of expression of the CD19 and CD22 antigens (Tables 1 and 5). Raji cells, which express the same levels of CD19 and CD22 as Daudi cells, had the same sensitivity to the IT-As. NALM-6 cells, which express about 1.5 times as much CD19 and one-fourth as much CD22 as Daudi cells, were about 10-fold less sensitive than Daudi cells to HD6-A and about twice as sensitive as Daudi cells to HD37-dgA (the only HD37-A tested). However, ARH-77 and NAMALWA cells, which express CD19 and CD22 at levels one-third to one-eighth those expressed on Daudi cells, were more than 50 times less sensitive to the IT-As than the Daudi cells. Thus, the sensitivity of the ARH-77 and NAMALWA lines to the IT-As was somewhat lower than would be expected from their levels of antigen expression. Jurkat cells, which do not express either CD19 or CD22, were resistant to both IT-As.

Effect of IT-As on Normal B-Cells. IT-As prepared from intact HD6 or HD37 or their Fab' fragments were highly cytotoxic to normal B-cells (Table 6) confirming earlier results (21). Similar IC_{50}s were obtained with IT-As (HD6) prepared either from intact antibodies or from their monovalent fragments, and the deglycosylation of A chain had little or no effect on the cytotoxicity of any of the IT-As. HD6-A ITs were only 5- to 10-fold less toxic than ricin. The cytotoxic effects of the IT-As were specific. The B-cells were not killed by MOPC-21-A at the highest concentrations tested.

Stability of HD6-A IT-As in Human Plasma. The toxicity of HD6 IT-As prepared from intact antibody or its $F(ab')_2$ and Fab' fragment was only marginally reduced by incubating the IT-As in fresh human plasma for 24 h at 37°C before testing them on Daudi cells (Table 7). A similar result was observed using HD6-IT-dgAs (data not shown). Thus, the IT-As are stable in human plasma at 37°C.

Effect of IT-As on Normal Human Bone Marrow. Previous studies on the distribution of CD19 and CD22 indicate that they are B-cell-restricted (15, 19). Since these antigens are expressed on fewer than 5% of bone marrow mononuclear cells, it is assumed that bone marrow hematopoietic progenitor cells lack CD19 and CD22 (13). These data, however, were based on immunofluorescence analyses. To extend these studies and confirm that anti-CD19-A and anti-CD22-A were not toxic to normal hematopoietic progenitor cells, bone marrow cells were treated with concentrations of IT-As (1 μg/ml) that normally inhibit protein synthesis in Daudi cells by greater than 90%. After treatment with the IT-As, the bone marrow cells were plated in soft agar and the growth of CFU-GM was determined. As shown in Table 8, no reduction of colony formation was seen when bone marrow cells were treated with either HD6-A or HD37-A. These studies indicate that HD6-A and HD37-A do not kill CFU-GM cells and suggest that these antigens

Table 5 *Cytotoxic activity of different HD6-, HD37-, and MOPC-21-derived IT-As on different human cell lines*

Cell type	ITs	Antibodies [IC_{50} (M)]		
		HD6	HD37	MOPC-21
ARH-77	IgG-A	$1.7 \pm 1.2 \times 10^{-9}$ (3)[a]	$1.5 \pm 0.7 \times 10^{-9}$ (3)	$>5.5 \times 10^{-8}$ (1)
	IgG-dgA	$6.0 \pm 3.0 \times 10^{-10}$ (3)	4.9×10^{-8} (2)	$>1.4 \times 10^{-7}$ (1)
	$F(ab')_2$-A	ND[b]	1.6×10^{-9} (2)	ND
	$F(ab')_2$-dgA	$2.0 \pm 1.2 \times 10^{-9}$ (3)	ND	ND
NAMALWA	IgG-A	ND	1.9×10^{-8} (1)	ND
	IgG-dgA	ND	3.1×10^{-8} (1)	$>4.2 \times 10^{-7}$ (1)
	Fab'-A	ND	3.4×10^{-8} (1)	ND
NALM-6	IgG-dgA	$1.6 \pm 0.5 \times 10^{-10}$ (3)	6.7×10^{-11} (1)	$>3.3 \times 10^{-8}$ (1)
	Fab'-dgA	4.0×10^{-10} (2)	ND	ND
Raji	IgG-dgA	3.0×10^{-11} (2)	1.6×10^{-10} (2)	$>3.3 \times 10^{-7}$ (2)
Jurkat	Fab'-A	1.0×10^{-7} (1)	1.0×10^{-7} (1)	$>1.0 \times 10^{-7}$ (1)

[a] Numbers in parentheses, number of experiments.
[b] ND, not determined.

Table 6 *Cytotoxicity of IT-As derived from HD6, HD37, and MOPC-21 on normal B-cells*

IT	Antibodies [IC_{50} (M)]		
	HD6	HD37	MOPC-21
IgG-A	$2.4 \pm 0.8 \times 10^{-10}$ (3)[a]	ND[b]	$>10^{-8}$ (3)
IgG-dgA	3.4×10^{-10} (2)	1.1×10^{-10} (1)	$>10^{-8}$ (3)
Fab'-A	$3.3 \pm 1.3 \times 10^{-10}$ (3)	$3.8 \pm 2.7 \times 10^{-9}$ (3)	$>10^{-7}$ (2)
Fab'-dgA	1.4×10^{-10} (2)	ND	$>10^{-8}$ (1)

[a] Numbers in parentheses, number of experiments. The IC_{50} value for ricin was $2.2 \pm 1.7 \times 10^{-11}$ M (5).
[b] ND, not determined.

Table 7 *Cytotoxicity of HD6-A IT-As on Daudi cells after their incubation with human plasma overnight at 37°C*

Incubation with	Cytotoxic activity[a]		
	IgG-A	$F(ab')_2$-A	Fab'-A
None	100	100	100
Plasma	72	85	83

[a] Mean of two experiments. The IC_{50} without treatment was considered 100%.

Table 8 *Soft agar cloning of human bone marrow cells following treatment with IT-As*

Experiment	Treatment	CFU-GM colonies[a]
1	Medium only	438 ± 134
	MOPC-21-A[b]	490 ± 63
	HD37-A[b]	604 ± 6
	HD6-A[b]	504 ± 167
2	Medium only	254 ± 12
	MOPC-21-A	324 ± 12
	HD37-A	244 ± 28
	HD6-A	290 ± 37

[a] Bone marrow cells were treated for 1 h at 37°C with concentrations of IT-A that reduced Daudi cell [^{3}H]leucine incorporation by 90% or greater (1 μg/ml). The cells were plated in duplicate in soft agar and the numbers of CFU-GM colonies (mean ± SD/10^5 bone marrow cells) were counted 14 days later.
[b] IgG (MOPC-21, HD37, HD6).

should also be absent from pluripotent stem cells. Thus, *in vivo*, administration of these ITs should not damage normal stem cells in the bone marrow.

Binding of HD6 and HD37 Antibodies to Normal Human Tonsils. The binding of HD6 and HD37 antibodies to tonsils was visualized using peroxidase-labeled rabbit anti-mouse immunoglobulin. Tonsils stained with both HD6 and HD37. As shown in Fig. 4, HD6 (*A*) gave stronger staining of the mantle zone whereas HD37 (*B*) gave stronger staining of germinal centers.

DISCUSSION

The major finding to emerge from this study is that IT-As constructed with the anti-CD22 antibody, HD6, are highly and specifically toxic to neoplastic B-cells and are more potent than IT-As constructed from the anti-CD19 antibody, HD37.

The greater potency of HD6-A as compared to HD37-A was most evident on Daudi cells to which the two antibodies bound in similar numbers and with similar affinities. IT-As prepared from intact HD6 antibody or its Fab' fragment had IC_{50} values 10-fold and 1.5- to 4-fold lower, respectively, than those of the corresponding HD37 IT-As. Importantly, treatment of the cells with saturating concentrations of the HD37-A did not reduce protein synthesis by more than 85%, indicating that a substantial proportion of the cells survived treatment. Under the same conditions, HD6-A abolished protein synthesis. The most likely explanation for the greater potency of HD6-A is that the CD22 antigen routes the IT-A to a compartment in the cell from which the A chain can translocate efficiently to the cytosol, whereas the CD19 antigen routes the IT-A to a compartment less favorable for A chain translocation, possibly the lysosomal compartment. In support of this suggestion, Press *et al.* (40) reported that IT-As directed against the CD3 and CD5, but not the CD2, antigen on human T-cells were cytotoxic. The differences in toxicity correlated with more rapid delivery to lysosomes and degradation of the ineffective IT-A.

Different neoplastic B-cell lines varied in their susceptibility to the HD6-A and HD37-A. The major factor determining susceptibility appears to be the concentration of the antigens expressed on the cells, as has been observed with other cell types (41). Daudi and Raji cells, which have high levels of both CD19 and CD22, were highly susceptible to both HD6-A and HD37-A; NALM-6 cells, which have low levels of CD22 but high levels of CD19, were about 10-fold less sensitive than Daudi or Raji cells to HD6-A and similar in sensitivity to HD37-A. However, other cell types such as NAMALWA and ARH-77, which have 3- to 8-fold lower densities of CD19 or CD22 on their surface than Daudi or Raji cells, were more than 50 times less susceptible than Daudi cells to both IT-As. This suggests that antigen density is not the sole factor which determines the susceptibility of a particular cell type to an IT-A, *i.e.*, as yet undefined characteristics of the cell type also determine its susceptibility. In this regard, Bjorn *et al.* (41) also showed that different breast cancer cell lines with similar densities of the same target antigens had completely different susceptibilities to IT-As.

Normal human B-cells were susceptible to IT-As prepared with HD6 and HD57 whereas bone marrow CFU-GM progenitor cells were resistant. This suggests that therapy with either HD6-A or HD37-A might cause immunosuppression (by killing normal B-cells) but should not cause damage to stem cells. Immunosuppression of patients is likely to be transient because new B-cells should soon emerge from precursor cells in the marrow, as has been demonstrated in the mouse (42). It might also extend the period of time that such IT-As can be administered before the patient raises neutralizing antibodies to either the mouse immunoglobulin or the ricin A chain.

Based on the data described in this report, our first Phase I/II clinical trials will utilize an anti-CD22-A. The overriding factor in reaching this decision was that anti-CD22-A has greater cytotoxic activity than anti-CD19-A on the majority of the neoplastic B-cell lines tested. However, the CD22 antigen is present at high levels on fewer types of B-cell neoplasms than the CD19 antigen (13–20) and this restricts the use of anti-CD22-A to the treatment of most B-cell lymphomas, some chronic lymphocytic leukemias, and a few rare tumors such as hairy cell leukemia. It must also be emphasized that the malignant stem cells in these diseases have not been identified and phenotyped, even though it is the destruction of these cells that is the primary objective of IT therapy. If the malignant progenitor cells are less mature than their more abundant progeny, they may express lower levels of CD22 antigens. However, these lower levels may still be sufficient for IT-A-mediated killing since the pre-B-cell leukemia line, NALM-6, which expresses low levels of CD22, is still highly susceptible to HD6-A.

In conclusion, based on the results of this preclinical comparison of different ITs *in vitro*, we plan to carry out a clinical trial to compare ITs (containing dg A chain) prepared with intact anti-CD22 antibody (linked via SMPT) to those made with its Fab' fragment (linked via Ellman's reagent). The anti-CD22-SMPT-dg A offers the advantage of high potency, longivity and stability (13) while the Fab'-anti-CD 22 dg A may

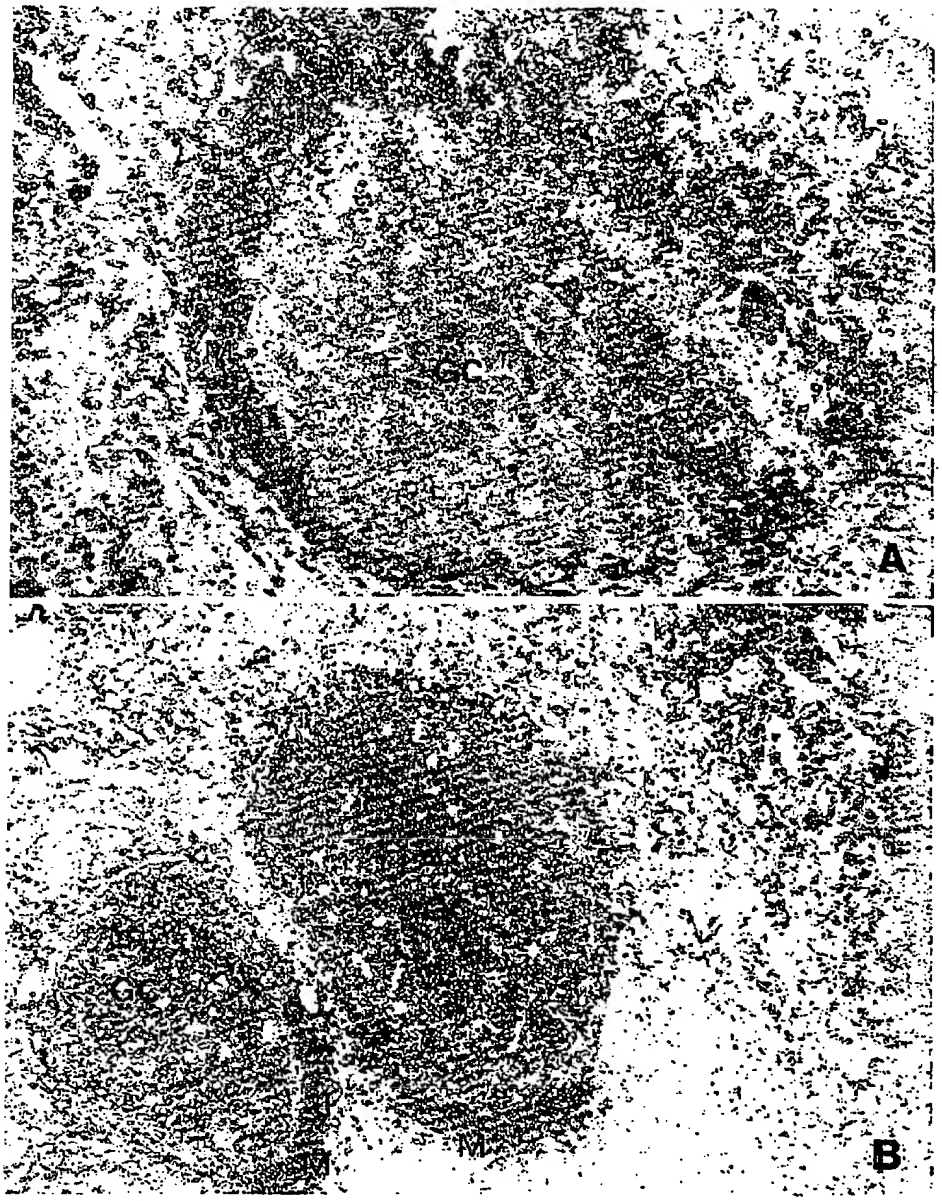

Fig. 4. Immunoperoxidase staining of a human tonsil. *A*, HD6 (anti-CD22) shows a single follicle in which the cells of the mantle (*M*) are darkly stained and more prominent than those of the germinal center (*GC*). *B*, HD37 (anti-CD19) shows two follicles and differs from HD6 in the more prominent staining of the GC cells.

be more effective in localizing to tumor tissue.[4] Thus, depending upon the type of B-cell tumor treated, each construct may offer different advantages.

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Abstract# 400 **Poster Board #-Session: 400-I**

REDUCED-DOSE ZEVALIN™ RADIOIMMUNOTHERAPY FOR RELAPSED OR REFRACTORY B-CELL NON-HODGKIN'S LYMPHOMA (NHL) PATIENTS WITH PRE-EXISTING THROMBOCYTOPENIA: REPORT OF INTERIM RESULTS OF A PHASE II TRIAL. Thomas E. Witzig,[1] Christine A. White,[2] Leo I. Gordon,[3] Russell J. Schilder,[4] Gregory A. Wiseman,[1] Amanda Rimmer*,[3] Elizabeth Parker*,[2] Antonio J. Grillo-Lopez.[2] *[1]Mayo Clinic, Rochester, MN; [2]IDEC Pharmaceuticals Corp., San Diego, CA; [3]Northwestern University, Chicago, IL; [4]Fox Chase Cancer Center, Philadelphia, PA.*

Zevalin (ibritumomab tiuxetan, IDEC-Y2B8) is an anti-CD20 murine IgG_1 kappa monoclonal antibody conjugated to tiuxetan (MXDTPA) which can securely chelate either ^{111}Indium (^{111}In) for imaging/dosimetry or ^{90}Yttrium (^{90}Y) for therapy. Phase I/II trials [Blood 1998;92 (10 Suppl):417a #1721 and #1722] demonstrated that clinical parameters of baseline platelet count and percent involvement of bone marrow with NHL were correlated with severity of hematologic toxicity while bone marrow dosimetry was not. MTD was found to be 0.4 mCi/kg (0.3mCi/kg in patients with mild thrombocytopenia). Based on this data, we conducted a Phase II trial to further evaluate the safety and efficacy of 0.3 mCi/kg of ^{90}Y labeled Zevalin for treatment of patients with relapsed or refractory, low-grade, follicular or transformed CD20+ B cell NHL. Patients with < 25 % bone marrow involvement (on bone marrow biopsy), no prior radioimmunotherapy, circulatory lymphocytes < 5000/mm^3, ANC ≥ 1500/mm^3, platelet counts between 100,000 and 149,000/mm^3, and no prior ABMT or stem cell therapy were eligible. Accrual is now complete with N = 30. Interim analysis was performed on the first 24 patients: median age 61 years (25% ≥ 75 years) ; 42% female; 83% follicular histology; 13% transformed; 4% small lymphocytic or lymphoplasmacytic; 46% had bulky disease ≥ 5cm, 21% had bulky disease > 7 cm; 13% had bulky disease ≥ 10 cm; 25 % had splenomegaly; 100% had prior chemotherapy (median prior therapies = 2); 13% had prior radiotherapy; and 4% had prior bioimmunotherapy. All patients underwent imaging and dosimetry with ^{111} Indium-labeled Zevalin. In all cases biodistribution and dosimetry were acceptable. (Protocol defined limits for estimated absorbed radiation dose were < 2000 cGy to normal organs and < 300 cGy to bone marrow). Toxicity was primarily hematologic, transient and reversible. Median nadirs for patients receiving 0.3mCi/kg (maximum dose 32 mCi) were ANC = 600/mm^3, platelets = 34,000/mm^3; and Hb = 10.0 gm/dl. Grade 4 neutropenia and thrombocytopenia occurred in 25% and 15 % of these patients respectively. Overall response rate was 68% (CR =23%; PR 45%) in the 22 patients for whom response assessment was available. Relapsed or refractory, low-grade, follicular or transformed CD20+ B cell NHL patients with mild thrombocytopenia can be safely treated with reduced-dose (0.3mci/kg) Zevalin with excellent clinical response.

Abstract# 401 **Poster Board #-Session: 401-I**

BIOLOGICAL RESPONSE OF B LYMPHOMA CELL LINES TO ANTI-CD20 MONOCLONAL ANTIBODY RITUXIMAB IN VITRO: CD55 AND CD59 REGULATE COMPLEMENT MEDIATED LYSIS. J. Golay,[1] L. Zaffaroni,[1] T. Vaccari,[1] G.-M. Borleri*,[2] F. Tedesco,[4] G. Dastoli,[3] T. Barbui*,[2] A. Rambaldi*,[2] M. Introna.[1] *[1]Istituto Mario Negri, Milano; [2]Division of Hematology, Ospedali Riuniti, Bergamo; [3]Roche Italia, Milano; [4]University of Trieste.*

Rituximab is a chimeric anti-CD20 monoclonal antibody which is being used successfully in the treatment of B cell Non-Hodgkin's lymphomas with a 30-50% response rate in relapsed patients. Its mechanism of action may include complement mediated and antibody-dependent cellular cytotoxicity, as well as a direct inhibition of proliferation and/or the induction of apoptosis. Here we have investigated its biological activity against 4 FL and 1 Burkitt's lymphoma (BL) cell lines as well as normal B cells in vitro. The results show that Rituximab can block the proliferation of normal B cells stimulated by SAC but had no effect on the proliferation of the lymphoma lines, nor did it induce B cell activation or apoptosis. Rituximab mediated ADCC against all cell lines to a similar extent. On the other hand human complement-mediated lysis was highly variable between cell lines, ranging from 100% lysis (DHL-4) to complete resistance (Karpas 422). Investigation of the levels of CD20 expression and of the complement inhibitors CD35, CD46, CD55 and CD59 suggested that CD55 is an important regulator of CDC. Blocking CD55 function with a specific antibody significantly increased CDC in the resistant lines. CD59 also plays a role in some lines since blocking CD59 also augments CDC. We conclude that CDC and ADCC are major mechanisms of action of Rituximab on B cell lymphomas, that a heterogeneous susceptibility of different lymphoma cells to complement may be at least in part responsible for heterogeneity of the response of different patients to Rituximab in vivo. Furthermore we show that the relative levels CD55 and CD59 may determine such a response.

Abstract# 402 **Poster Board #-Session: 402-I**

EXCELLENT TOLERANCE OF RITUXAN WHEN GIVEN AFTER MITOXANTRONE-CYCLOPHOSPHAMIDE: AN EFFECTIVE AND SAFE COMBINATION FOR INDOLENT NHL. Christos Emmanouilides*,[1] Milhan Teletar*,[2] Peter Rosen*,[1] Harry Menco*,[3] Ravi Patel*,[3] John Barstis*,[3] Rose Malone*,[1] Mary Territo.[1] *[1]Division of Hematology-Oncology, UCLA, Los Angeles, CA; [2]Department of Pathology, UCLA, Los Angeles, CA; [3]UCLA Oncology Network, Los Angeles, CA.*

Treatment for extensive indolent lymphoma should combine optimization of efficacy with reduction of toxicity. Rituxan may be an ideal agent for combinations with chemotherapy because of non-cross resistant efficacy and differential toxicity; however, lethal complications have occured. Furthermore, the potential for synergism with chemotherapy has been documented. 24 patients with indolent B-cell NHL have been treated to-date utilizing a novel three drug combination. Pt characteristics: median age:60, (range 36-72); histology: follicular 13, SLL/CLL 6, lymphoplasmacytic 4, marginal 1; all pts had stage III or IV disease; 10 pts had prior treatment with 1-3 courses of chemotherapy (including alkylating agents). Patients first received cyclophosphamide 800 mg/m^2 and mitoxantrone 8 mg/m^2, IV on day 1, every 3 weeks for 2 cycles. Subsequently, patients received rituxan 375 mg/m^2 followed by mitoxantrone 8 mg/m^2 every 2 weeks for 4 cycles. The regimen and, particularly, rituxan were extremely well tolerated. Only 2 pts out of 22 assessable experienced a grade II, infusion-related toxicity (mild chills, T:37.9 or rash) during the first rituxan treatment (one patient had received prior rituxan). Grade IV neutropenia was noted at some point in 14 pts who were offered GMCSF support for improvement of neutropenia and, possibly, enhancement of ADCC. No infections were noted. Alopecia was minimal. Out of 21 evaluable patients, 1 had a transient PR, 3 have a sustainable PR and 17 are in CR after a median follow-up of 6 months. Hence, the overall response rate is 95%. The objective responses per subtype are follicular: 11/11, SLL: 4/5, plasmacytic: 4/4, marginal: 1/1. Except for one, all responders remain in remission although the follow-up is short. Molecular remissions were noted in 3 of 5 tested patients. We conclude that the cyclophosphamide-mitoxantrone-rituxan (CyMiR) regimen is effective and extremely well tolerated. Furthermore, rituxan-related morbidity has not been seen.

Abstract# 403 **Poster Board #-Session: 403-I**

ZEVALIN™ BIODISTRIBUTION AND DOSIMETRY ESTIMATED NORMAL ORGAN ABSORBED RADIATION DOSES ARE NOT AFFECTED BY PRIOR THERAPY WITH RITUXIMAB. Gregory A. Wiseman,[1] Christine A. White,[2] William Erwin*,[3] Dominic Lamonica,[4] Ellen Kornmehl*,[5] Daniel H. Silverman*,[6] Thomas E. Witzig,[1] Leo I. Gordon,[3] Marcelina E. White*,[2] Richard Belanger*,[7] Antonio J. Grillo-Lopez.[2] *[1]Mayo Clinic, Rochester, MN; [2]IDEC Pharmaceuticals, San Diego, CA; [3]Northwestern University, Chicago, IL; [4]Roswell Park Cancer Center, Buffalo, NY.*

Imaging and dosimetry are performed in advance of radioimmunotherapy to ensure acceptable biodistribution and absorbed radiation dose delivered to normal organs. Zevalin (IDEC-Y2B8, ibritumomab tiuxetan) is an anti-CD20 murine IgG_1 kappa monoclonal antibody conjugated to the linker tiuxetan, which can securely chelate either ^{111}Indium for imaging/dosimetry or ^{90}Yttrium for therapy. Zevalin is given with Rituximab (250 mg/m^2 is given prior to each Zevalin dose) to clear peripheral blood B lymphocytes and optimize Zevalin tumor targeting. Phase I/II results [Blood 1998;92(10 Suppl):417a #1721 & #1722] have previously demonstrated that a standard dose (0.4 mCi/kg) of ^{90}Y labelled Zevalin could be used to treat patients with B-cell NHL with acceptable dosimetry and clinical safety, and excellent efficacy (ORR 67% in low, intermediate-grade, and mantle cell NHL, 82% in low-grade patients; median time to progression 12.7 months in responders; 23.8+ months in patients treated at 0.4 mCi/kg who achieved a CR). That trial also demonstrated that the clinical parameters of baseline platelet count and percent NHL involvement of bone marrow were predictors for severity of hematologic nadir, but dosimetry was not correlated with severity of hematologic nadir. A Phase III non-randomized controlled trial of Zevalin therapy in Rituximab refractory [non-responders or < 6 months TTP] patients is now ongoing. We performed imaging and dosimetry on 27 patients who were refractory to prior Rituximab to ensure that biodistribution and absorbed radiation dose to normal organs were not adversely effected by prior therapeutic doses (375mg/m^2 weekly x4) of Rituximab. Protocol defined acceptable dose as < 2000 cGy estimated absorbed radiation dose to normal organs and < 300 cGy to red marrow. Patients underwent imaging and dosimetry during the week prior to Zevalin therapy (0.4 mCi/kg). Radiation dose estimates were calculated at each investigative site with the MIRDOSE 3.1 code using observed biokinetics of ^{111}In labeled Zevalin. Biodistribution and dosimetry were acceptable in all 27 cases. Organ dosimetry summary statistics are available on the first 24 patients. Median estimated absorbed radiation doses to normal organs were: 14.6 cGy/mCi to liver; 7.0 cGy/mCi lung; 7.6 cGy/mCi to kidney; 3.0 cGy/mCi to red marrow; and 2.3 cGy/mCi to total body. As previously demonstrated in the Phase I/II study there was no correlation between estimated bone marrow absorbed radiation dose and severity of ANC or platelet nadir. This interim data demonstrates that Zevalin biodistribution and dosimetry was not affected by prior Rituximab courses (375 mg/m^2 x 4 wks) given 2-23 months earlier.

Abstract# 404 **Poster Board #-Session: 404-I**

EPRATUZUMAB, A NEW ANTI-CD22, HUMANIZED, MONOCLONAL ANTIBODY FOR THE THERAPY OF NON-HODGKIN'S LYMPHOMA (NHL): PHASE I/II TRIAL RESULTS. John P. Leonard,[1] Morton Coleman,[1] Michael W. Schuster,[1] Amy Chadburn*,[1] Scott Ely*,[1] Neda Yagan*,[1] Robert M. Sharkey*,[2] Hans J. Hansen*,[2] David M. Goldenberg*,[2] *[1]Center for Lymphoma and Myeloma, Division of Hematology/Oncology and Departments of Pathology and Radiology, Weill Medical College of Cornell University and New York Presbyterian Hospital, New York, NY; [2]Garden State Cancer Center, Belleville, NJ; [3]Immunomedics, Inc., Morris Plains, NJ.*

Epratuzumab is a humanized anti-CD22 monoclonal antibody IgG1 (hLL2; LymphoCide™, Immunomedics, Morris Plains, NJ) that has been studied in radiolabeled forms (^{131}I and ^{90}Y) in the treatment of chemotherapy refractory NHL. The aim of this trial is to assess the safety and efficacy of escalating doses of the naked (unlabeled) form of epratuzumab in NHL patients who relapsed after chemotherapy. At least 6 patients (3 indolent and 3 aggressive) were treated with 4 weekly infusions of epratuzumab at each of 5 dose levels, ranging from 120 mg/m^2 to 600 mg/m^2 per injection. To date, 30 patients have been enrolled, with 23 currently assessable for toxicity and response. Dose-limiting toxicity has not been observed, and the 1-hour infusions have been tolerated well at all dose levels. No drug-related grade 3 or 4 toxicity has been observed; only grade 2 hypotension (n=2) and grade 1 rigors and fatigue (n = 3 each) were noted primarily during the initial infusion. All other drug-related events were only grade 1, including blood count or chemistry changes. Serum immunoglobulin levels have not been affected, nor has there been evidence of pharmacokinetic changes following repeated injections. There is no evidence of a significant anti-human antibody response. Reduction of circulating CD22-positive cells has been observed in several patients. No responses were seen at dose level 1 (120 mg/m^2/week); one diffuse large cell lymphoma patient treated at dose level 2 (240 mg/m^2/week), who previously relapsed after chemotherapy and autologous stem cell transplant, and who was also unresponsive to rituximab therapy, has an ongoing complete remission for over 1 year. Two of 3 indolent NHL patients treated at dose level 3 (360 mg/m^2/week) were responders (1 CR, 1 PR), as well as 2 of 3 indolent patients at dose level 4 (480 mg/m^2/week)

(1 CR, 1 PR). Four of these 5 responses are ongoing. Epratuzumab levels were detectable in the serum for up to 3 months after treatment, and patients with objective responses had higher circulating levels. Epratuzumab is well tolerated across a wide range of protein doses given as a 1-hour infusion, and can result in objective tumor responses, suggesting that the antibody is a potential new therapy for NHL that may be effective in rituximab failures. Further dose escalation in larger numbers of patients is ongoing to determine the optimal dose and response rate.

Abstract# 405 **Poster Board #-Session: 405-I**

MANIPULATION OF CD22 SIGNAL TRANSDUCTION FOR THE TREATMENT OF LYMPHOMA. J. M. Tuscano,[1] G. DeNardo*,[1] T. Wun,[1] T. F. Tedder*,[2] J. H. Kehrl.[3] *[1]Internal Medicine, UC Davis Cancer Center, Sacramento, CA; [2]Immunology, Duke University, Durham, North Carolina; [3]Immunoregulation, NIAID, Bethesda, MD.*

The humoral immune response depends upon a highly specific interaction between antigen and B lymphocyte antigen receptor (BCR). This interaction initiates signal transduction cascades necessary for B-cell development and differentiation. CD22 is a membrane glycophosphoprotein found on nearly all B lymphocytes and most B-cell lymphomas. Crosslinking CD22 triggers CD22 tyrosine phosphorylation and assembles a complex of effector proteins that activate the stress-activated protein kinase (SAPK) pathway. In conjunction with interleukins, antigen receptor crosslinking or CD40 crosslinking; CD22 crosslinking provides a potent costimulatory signal in primary B-cells and pro-apoptotic signal in neoplastic B-cells. Recent studies have revealed several anti-CD22 monoclonal antibodies (mAbs) with distinct functional properties (termed HB22.7, HB22.23, and HB22.33). Crosslinking CD22 on primary and several B cell lymphoma cell lines with these mAb's results in a 3-5 fold induction of SAPK activity. Apoptotic analysis of Ramos cells by flow cytometric means revealed a 48.5% and 82.2% induction of apoptosis when CD22 was crosslinked with HB22.7 and HB22.33 respectively, compared to 8.7% with a control mAb. Titration of the pro-apoptotic effects of these mAb's revealed that each mAb induced a similar degree of apoptosis, however at significantly different concentrations, with 82% of Ramos cells apoptotic with 80ug/cc of HB22.7, and a similar degree of apoptosis observed with only 10ug/cc of HB22.33. A similar degree of apoptosis was observed when CD22 was crosslinked on the lymphoma cell lines ST-486 and BL41. Xenograft-specific targeting was demonstrated utilizing ^{111}In-conjugated HB22.7 in Ramos and Raji xenograft-bearing nude mice models. The Raji model demonstrated significantly better HB22.7 targeting which is consistent with a Scatchard analysis of both the Ramos and Raji cell lines that demonstrated 105 and 106 antibodies/cell respectively. Next a pilot study was done to examine the therapeutic potential of HB22.7 in Raji nude mice xenografts. After 21 days, when compared to untreated control mice, HB22.7-treated mice demonstrated a 5 fold reduction in tumor volumes. Based on the ability to induce the SAPK pathway, induce apoptosis, shrink lymphoma xenografts and relatively low doses, of these mAb's have significant therapeutic potential.

Abstract# 406 **Poster Board #-Session: 406-I**

ACTIVE IMMUNIZATION USING DENDRITIC CELLS MIXED WITH TUMOR CELLS INHIBITS THE GROWTH OF LYMPHOMA. J. Park*,[1] S. H. Kim,[1] C. Suh,[1] J. H. Yang*,[2] T. W. Kim*,[1] J. H. Lee*,[1] S. B. Kim*,[1] S. W. Kim*,[1] K. H. Lee,[1] J. S. Lee,[1] W. K. Kim.[1] *[1]Hematology/Oncology, Asan Medical Center, Seoul, Republic of Korea; [2]Asan Institude for Life Science & Technology, Seoul, Republic of Korea.*

Dendritic cells(DCs) are potent antigen-presenting cells for the induction of cytotoxic T lymphocytes and have proven to be effective immunogens when pulsed with tumor associated antigens. The aim of our study was to test whether bone marrow derived DCs are capable of inducing protective immunity against a murine lymphoma (A20). DCs were grown from tumor bearing Balb/c mice by culturing bone marrow in murine GM-CSF, IL-4 and TNF-α containg medium for 12 days. DCs with irradiated tumor cells showed a significantly increased stimulation of allogenic T cells (p=0.006) and increased cytotoxic T cell responses than DCs alone *in vitro*. For evaluation of the effect of immunization on the growth of the established tumor, Balb/c mice were injected sc with 2 x 10^6 A20 cells (group A) or 5 x 10^6 A20 cells (group B). Intra peritoneal immunization with 2 ~ 4 x 10^5 DCs mixed with 2 x 10^6 lethally irradiated (10,000 rad) A20 cells were started when the tumor reached 4 ~ 5 mm in diameter (group A) or on day -7 (group B). Booster intraperitoneal immunization were given every 3 ~ 4 days for 3 weeks. Mice in control groups were given intraperitoneal inoculations of phosphate buffered saline solution (PBS), 2 ~ 4 x 10^5 DCs or lethally irradiated A20 cells alone. For evaluation of the effect of immunization on T cells, [^{3}H]thymidine uptake test, FACS scan analysis of T cells and IL-2/IL-4 assay (using an ELISA) with splenocytes of each groups were used.

	Immunization group	Tumor volume (mm^3) at 28 days	CD4/CD8(%)	^{3}H uptake test (CPM)	IL-2 / IL-4 (pg/ml)
A. 2 x 10^6 A 20 cells	PBS	5301±3493	28/8	4045±796	130 / 81
	DCs	2485±1729	33/22	26324±2301	2229 / 1144
	DCs + A20	1945±1125*	46/17	1795±1935	1535 / 1150
B. 5 x 10^6 A 20 cells	PBS	418±323			
	DCs + A20	no tumor growth+			
	Normal control		31/13	128±53	116/70

* $P < 0.05$, + no tumor growth after completion of immunization.

These results suggest that DCs mixed with tumor cells as a source of undefined tumor antigens can induce an effective antitumor immune response. And complete prevention of tumor growth when injection of low dose tumor cells provides a rationale for the use of DCs and tumor cells in immunotherapy of minimal residual disease of lymphoma.

Abstract# 407 **Poster Board #-Session: 407-I**

CLINICAL PHASE I/II TRIAL WITH A NOVEL RICIN A-CHAIN IMMUNOTOXIN (Ki-4.dgA) IN PATIENTS WITH REFRACTORY CD30+ LYMPHOMA. Roland Schnell*,[1] J. Oliver Staak*,[1] Christine Schwartz*,[1] John Schindler,[2] Volker Diehl,[1] Ellen Vitetta,[2] Victor Ghetie,[2] Andreas Engert.[1] *[1]Clinic I for Internal Medicine, University of Cologne, Cologne, Germany; [2]Cancer Immunobiology Center and Department of Microbiology, The University of Texas, Southwestern Medical Center, Dallas, TX.*

Immunotoxins (ITs) consisting of a cell-binding moiety and a potent toxin were developed as a new class of biological anti-tumor agents to improve adjuvant therapy. Hodgkin's lymphoma (HL) are an excellent target for IT treatment due to the expression of lymphocyte activation markers such as CD30. We constructed an anti-CD30 Ki-4.dgA IT by linking the monoclonal antibody Ki-4 to deglycosylated ricin A-chain. *In vitro*, Ki-4.dgA was five-fold more potent compared to other anti-CD30 A-chain ITs and demonstrated high anti-tumor activity in a HL SCID-mice model. Therefore, Ki-4.dgA was selected for clinical use. Objectives of this trial are the evaluation of the maximum tolerated dose (MTD), assessment of dose limiting toxicities, analysis of pharmacokinetic parameters and immune response against the IT as well as documentation of biologic effects or clinical response. As of 8/99 nine patients were enrolled in this ongoing trial. Eight patients presented with HL and one with CD30+ large cell anaplastic lymphoma (Ki-1 lymphoma). All pts. were heavily pretreated with a median of six different prior therapies including autologous bone marrow transplantation in seven of nine. The mean age was 34 years (range 26 to 52). 7/9 patients had advanced disease (stage IIIB/IV) and 3/9 had B-symptoms at study entry. Five patients had primarily progressive disease (remission < three months). The mean time from initial diagnosis was 6.6 years. The IT was administered i.v. over four hours on days 1-3-5-7 for total doses per cycle of 5, 7.5 or 10 mg/m^2. Patients received one to three cycles. Side effects were reversible and related to the vascular leak syndrome (VLS), i.e. decrease in serum albumin, edema, weight gain, hypotension, tachycardia, myalgia and weakness. No hematologic toxicity was observed. 3/9 patients demonstrated a grade I allergic reaction. One of six patients treated at 5 mg/m^2 experienced NCI grade III toxicity (fatigue syndrome), all other patients tolerated the treatment at this dose level well. In both patients receiving 10 mg/m^2 NCI ≥ grade III toxicities occurred (weight gain, fatigue syndrome, myalgia). Thus, we reduced the dosage to 7.5 mg/m^2 for further treatment and the MTD will be defined at 5 or 7.5 mg/m^2. 25% of the patients developed human anti-ricin antibodies and 12.5% human anti-mouse antibodies. The pharmacokinetic and immunologic data will be reported later. Clinical results included two minor responses, two stable disease and five progressive disease. Further patients need to be treated to investigate the IT's clinical use and efficacy.

Abstract# 408 **Poster Board #-Session: 408-I**

RECOMBINANT TOXINS CONTAINING PSEUDOMONAS EXOTOXIN DIRECTLY INJURE HEPATOCYTES IN THE ABSENCE OF KUPFFER CELLS. Gregory Hoestand*,[1] David H. Robbins*,[1] Masanori Onda*,[1] Ira Pastan*,[1] Robert J. Kreitman.[1] *[1]Laboratory of Molecular Biology, National Cancer Institute, NIH, Bethesda, MD.*

Liver toxicity is dose-limiting in the administration of anti-Tac(Fv)-PE38 (LMB-2), a recombinant anti-CD25 single-chain immunotoxin which has resulted in major responses in patients with chemotherapy-refractory hairy cell leukemia, chronic lymphocytic leukemia, adult T-cell leukemia, cutaneous T-cell lymphoma, and Hodgkin's disease. Recent experiments have indicated that Pseudomonas exotoxin (PE) causes liver toxicity in mice via mechanisms which depend on TNFα, T-cells, and Kupffer cells (Schumann et al., J. Immunol, 161:5745, 1998). We found that the anti-CD22 recombinant immunotoxin RFB4(dsFv)-PE38 (BL22), which contains the same truncated toxin as LMB-2, is much less toxic to mice due to reduced liver toxicity. Thus the mechanism for differential liver toxicity is unrelated to specific binding, since murine CD25 and CD22 are incapable of binding LMB-2 and BL22. The differential hepatic toxicity of BL22 and LMB-2 is not limited to mice, as it is also observed in monkeys. To determine whether hepatic parenchymal cells are sensitive to the direct cytotoxic effect of recombinant toxins, parenchymal hepatocytes from mice and rats were isolated by collagenase perfusion and exposed to recombinant toxins. In the absence of Kupffer cells and TNFα (<5.1pg/ml by ELISA), LMB-2 and BL22 were cytotoxic to murine hepatocytes with IC50s, as measured by leucine incorporation, of 150 +/- 10 and 650 +/- 115 ng/ml, respectively. The cytotoxicity correlated with apoptosis of the parenchymal hepatocytes. At 37C for 6 hours the immunotoxin nonspecifically taken up by murine hepatocytes was directly proportional to time ($r2 = 0.90$-0.95) and amounted to 1080 +/- 5 and 260 +/- 5 molecules/cell/min for LMB-2 and BL22, respectively. The difference in isoelectric point (pI) of the ligands for LMB-2 and BL22 (10.2 vs. 7.67, respectively) suggested that interaction of basic regions of the protein may play a role in uptake by liver. To investigate this hypothesis, a number of different recombinant immunotoxins and growth-factor fusion-toxins were also incubated with hepatocytes isolated from mice, rats, monkeys and humans. Using four different fusion proteins containing a 15 kDa growth factor fused to PE38, cytotoxicity was directly proportional to the pI of the ligand, with r2 values of 0.85, 0.95, 0.9, and 0.8 for murine, rat, Cynomolgus monkey, and human hepatocytes, respectively. Significant but lower correlations of cytotoxicity with ligand pI were observed in comparing the cytotoxicity of a variety of 63 kDa recombinant immunotoxins. We conclude that while indirect cytokine-related mechanisms may be involved, recombinant toxins containing truncated PE are directly cytotoxic to hepatocytes, at concentrations which are typically achieved in the plasma of treated patients. Our experiments support the hypothesis that basic charges on the ligand lead to nonspecific interaction with hepatocytes, and support efforts to decrease the pIs of ligands used to make recombinant toxins.

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Potentiation by Interleukin 2 of Burkitt's Lymphoma Therapy with Anti-Pan B (Anti-CD19) Monoclonal Antibodies in a Mouse Xenotransplantation Model

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ABSTRACT

To study the immunotherapeutic potential of monoclonal antibodies (mAbs) directed against the human pan-B-cell antigen CD19, a xenotransplantation model was developed in which the human Burkitt's cell line Daudi is s.c. transplanted into nude mice.

IgG1, IgG2b, and IgG2a isotype variants of the anti-CD19 mAb (CLB-CD19) were tested for their capacity to inhibit the growth of 10×10^6 Daudi cells injected s.c. into nude mice. When mAb treatment was started 30 min after the injection of tumor cells, only the IgG2a isotype of CLB-CD19 had a marked antitumor effect *in vivo*. If treatment with IgG2a anti-CD19 mAb alone was delayed until Day 10 after tumor injection, no therapeutic effect was observed. However, the combination of this delayed mAb treatment with recombinant Interleukin 2 (rIL-2) inhibited the growth of the Daudi cells in the nude mice, while treatment with rIL-2 alone was ineffective.

The results of *in vitro* experiments showed that peritoneal exudate cells were able to inhibit the proliferation of Daudi cells in the presence of the IgG2a isotype variant of CLB-CD19 mAb but not in the presence of the other CLB-CD19 mAb isotype variants.

Fresh nude mouse spleen cells did not mediate antibody-dependent cellular cytotoxicity against CLB-CD19 mAb-sensitized Daudi cells, irrespective of the isotype used for sensitization. However, preculture of these spleen cells with rIL-2 induced antibody-dependent cellular cytotoxicity against $CD19^+$ target cells sensitized with CLB-CD19 mAb of all isotypes.

These results indicate that it is possible to enhance mAb-dependent effector systems *in vivo* with the lymphokine rIL-2.

INTRODUCTION

The use of mAbs[2] for the therapy of malignant disease continues to draw wide attention. Phase I/II clinical trials with mAbs have, *e.g.*, been performed in patients with melanoma (1), colon carcinoma (2, 3), or leukemia/lymphoma (4–9). Although the clinical effects were limited, the results encourage further study with the ultimate goal to improve the efficacy of mAb therapy.

The therapeutic effect of mAbs as such, *i.e.*, not coupled to toxins or radioisotopes, depends on the recruitment of host effector systems, including complement, ADCC, and phagocytosis and/or cytostasis of antibody-coated tumor cells (10–13). Thus, optimal utilization of the therapeutic potential of mAbs requires optimal mobilization of these effector systems.

The importance of the mAb isotype used for immunotherapy has been most elegantly studied with isotype switch variants of mouse mAb (14–16). In these studies, mouse IgG2a was consistently identified as the most effective isotype in depleting antigen-positive cells *in vivo* and in directing mouse and human effector cells to mediate ADCC activity *in vitro*. However, although the mouse IgG3 isotype of a series of isotype switch variants was not very effective in mediating ADCC (14), IgG3 mAbs directed against a human melanoma-associated antigen were described as very effective in a clinical trial in melanoma patients (1), in inhibiting the growth of human melanoma in nude mice, and in directing mouse and human effector cells to mediate ADCC activity *in vitro* (17).

Rat mAbs have been advocated as more active reagents than mouse mAbs for the treatment of malignant disease (18, 19). Of the rat immunoglobins, IgG2b was shown to be very efficient in directing human and mouse effector cells to mediate ADCC and in activating complement (18), although for complement activation, not only the mAb isotype but also the structure and density of the recognized antigen were shown to be of importance (20).

The limited efficacy of mAbs in clinical trials may in part be explained by a lack of optimally activated host effector systems that are potentially able to clear mAb-coated tumor cells. Biological response modifiers such as INF and IL-2 are known to enhance cellular and humoral immune responses (21, 22) and therefore offer a possibility to overcome this problem. Indeed, it was shown recently that rIL-2 can increase the ADCC activity of mouse (23, 24) and human (25, 26) effector cells. Moreover, a synergistic antitumor effect was observed *in vivo* with the combined treatment of experimental animal tumors with mAbs and INF-α (27) or rIL-2 (23, 28–30).

Antibodies directed against differentiation antigens expressed on lymphocytes are possible candidates for the treatment of leukemia/lymphoma. The results of clinical trials with mAbs directed against the differentiation antigens CD5 or CD20 have been published (7–9). In both systems tumor responses were observed, and side effects that accompanied mAb infusions were mild. Another possible target antigen for immunotherapy of B-cell malignancies is CD19, a B-cell-specific antigen present on most differentiation stages. Recently, a detailed analysis of the CD19 expression on leukemia or lymphoma cells was published (31). This study showed that 88% of B-lineage lymphoma cases and 100% of B-lineage leukemia cases expressed CD19. Importantly, CD19 expression was observed on the putative leukemia stem cells of three B-cell precursor acute lymphocytic leukemia cases.

In order to test the immunotherapeutic potential of the anti-CD19 mAb CLB-CD19 (32), we developed a xenotransplantation model in which human Burkitt's lymphoma cell lines are transplanted in nude mice. We present here the encouraging results of immunotherapy experiments, performed in this model with isotype switch variants of CLB-CD19 mAb and with the combination of IgG2a CLB-CD19 mAb and rIL-2. The results of this study suggest that it is possible to enhance the effect of mAbs *in vivo* with rIL-2, thus providing a rationale for clinical

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[2] The abbreviations used are: mAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; E/T, effector/target cell ratio; FCS, fetal calf serum; INF, interferon; 2ME, mercaptoethanol; NBCS, newborn calf serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; SDS, sodium dodecyl sulfate; rIL-2, recombinant interleukin 2; FITC, fluorescein isothiocyanate; NK, natural killer; FcR, Fc receptor.

trials in patients with B-cell malignancies with anti-CD19 mAb and rIL-2.

MATERIALS AND METHODS

Animals. C3H-*nu/nu*, C57BL/6 Kh-*nu/nu* BALB/c mice, and R rats were bred and maintained under specific-pathogen-free conditions at the animal department of the Netherlands Cancer Institute. Male and female BALB/c-*nu/nu* mice were purchased from: TNO, Zeist, The Netherlands; Bomholtgart, Ltd., Ry, Denmark, or Harlan-Olac, Ltd., Blackthorn, England. For *in vivo* experiments, male or female nude mice were used between 4 and 8 wk of age and maintained in sterile isolators. The mice used as effector cell sources for *in vitro* experiments were between 1 and 4 mo of age.

Cell Lines and Cell Culture Conditions. The human Burkitt's cell lines Namalwa, Ramos, Raji, EB3, Daudi, and Jiyoye were purchased from the American Type Culture Collection, Rockville, MD. The JVM3 cell line was derived from the tumor cells of a B-prolymphocytic leukemia patient after Epstein-Barr virus transformation *in vitro* (33). The rat mycloma cell line IR983F and the mouse anti-rat κ-producing hybridoma MARK-1 (34) were kindly provided by Dr. Bazin (Belgium). The cell lines were cultured in DMEM supplemented with 2 g of glucose/liter, 1 mM sodium pyruvate, 4 mM L-glutamine, nonessential amino acids, 100 IU penicillin/ml, 100 μg of kanamycin/ml, and NBCS (10%, v/v) (Sera Lab., Sussex, England), or FCS (10%, v/v) (PAA Lahorgesellschaft, Austria). Murine cells and cell lines were cultured in DMEM/FCS supplemented with 5×10^{-5} M mercaptoethanol (DMEM/FCS/2ME).

Monoclonal Antibodies. The production, isolation, and characterization of isotype switch variants of the anti-CD19 mAb (CLB-CD19) were previously described (32).

R24.3 mAb was produced as previously described (35), except that as fusion partner for the rat spleen cells, the rat myeloma cell line IR983F was used. The antigen recognized by R24.3 mAb was identified as HLA Class II by immunoprecipitation followed by SDS/PAGE analysis (36, 37). The R24.3 mAb was determined to be of the IgG2b isotype using the Ouchterlony double-diffusion procedure with rabbit anti-rat immunoglobulin class- and subclass-specific antibodies (Nordic, Tilburg, The Netherlands). The irrelevant control mAbs used in *in vivo* and *in vitro* experiments were: K8, S6, NKI-beteb, mouse IgG2a, IgG1, and IgG2b mAbs, respectively; and the rat IgG2b mAb 50B8. The NKI-beteb mAb was obtained from Dr. C. Vennegoor, Amsterdam, and the 50B8 mAb was kindly provided by Dr. A. Sonnenberg, Amsterdam, The Netherlands.

Purification of mAbs. Initially the mAbs MARK-1, K8, and the heavy-chain isotype variants of CLB-CD19 were isolated from ascites on a preparative high-performance liquid chromatography column (Bakerbond mAb; J. T. Baker, Inc., NJ). In later purification procedures, the Bakerbond AbX (Baker) column matrix was used according to a previously described procedure (38). The purity of mAbs was estimated on the basis of SDS/PAGE analysis (37) and was consistently between 90 and 95%. Purified mAbs were dialyzed for 24 h against PBS and stored at −20°C until use. mAbs used for *in vivo* immunotherapy experiments were supplemented with 0.5% BSA (w/v) (Fraction V; Sigma) and sterilized by filtration (Millipore 0.22-μm diameter pore).

Isolation and Culture of Murine Spleen Cells and Mouse Peritoneal Exudate Cells. Spleens were excised under aseptic conditions and immediately transferred to approximately 10 ml of DMEM/FCS/2ME. The spleens were subsequently minced using a plastic mesh filter (NPBI BV; Emmer-Compascuum, The Netherlands), and the resulting cell suspension was washed once with medium and reconstituted at the desired density in DMEM/FCS/2ME. BALB/c nude mouse spleen cells were IL-2 activated by incubating them for 6 days in DMEM/FCS/2ME supplemented with 500 units of rIL-2/ml (2 to 3×10^6 cells/ml). After this culture period, dead cells and erythrocytes were removed by centrifugation over Ficoll-Hypaque (Lympholyte M; Cedarland Lab., Ltd., Ontario, Canada). Peritoneal exudate cells were obtained from thioglycolate-treated (Oxoid, London, England) BALB/c mice by washing their peritoneal cavities with 5 ml of PBS. The harvested PEC were immediately transferred to ice-cold NBCS, washed once with medium, and reconstituted at the desired cell density in DMEM/FCS/2ME.

Immunofluorescence. Cells were incubated in the appropriate dilutions of mAbs (5×10^5 cells in 50 μl). Bound mouse immunoglobulin was detected with FITC-conjugated $F(ab)_2$ fragments of goat anti-mouse immunoglobulin/FITC (Tago, Burlingame, CA). Bound rat immunoglobulin was detected with biotinylated mouse anti-rat κ (MARK-1) followed by an incubation with avidin/FITC (Vector, Burlingame, CA). Biotinylation of MARK-1 mAbs was performed as previously described (39). All incubations were performed for 30 min at 0°C, and after each incubation, the cells were washed once with 2 ml of PBS supplemented with 0.5% (w/v) BSA and 0.1% (w/v) sodium azide (PBS/BSA/azide). Fluorescent staining was analyzed with a FACSCAN cytofluorimeter (Becton and Dickinson, Mountain View, CA) or using a fluorescence microscope, screening at least 200 cells.

The intact antibody content of purified CLB-CD19 mAbs and the plasma levels of CLB-CD19 mAbs were determined by indirect immunofluorescence. Mean fluorescence intensity was interpolated on a standard curve of intensities obtained with known concentrations of CLB-CD19 mAb.

***In Vivo* Experiments.** Human Burkitt's cell lines used for *in vivo* experiments were grown *in vitro*, washed once with medium, and reconstituted at the desired cell density in PBS. These cells were injected s.c. or i.p. (0.2 ml/injection) into nude mice, and the mice were monitored at regular intervals thereafter. Once a week the s.c. developing tumors were measured with precision calipers. These measurements were expressed as the product of two perpendicular diameters (tumor area, mm^2).

In immunotherapy experiments, the take-rate (number of mice with tumor/total number of mice) was recorded on Day 60, and a statistical analysis was performed on the sum of the take-rates of independent experiments with the Fisher exact test. Differences were considered significant if $P \leq 0.05$. Therapy experiments with isotype switch variants of CLB-CD19 mAb were performed as follows. On Day 0, BALB/c-*nu/nu* mice received 3 Gy of whole-body irradiation prior to the s.c. injection of 10×10^6 Daudi cells. The mAb was given i.p. [1 mg/injection in 1 ml of PBS, 0.5% (w/v) BSA] on Days 0, 3, and 6. Control animals received 1 ml of PBS/BSA or 1 mg of irrelevant mAb (K8 antiidiotype, IgG2a isotype) in 1 ml of PBS/BSA on Days 0, 3, and 6.

Combined treatment with IgG2a CLB-CD19 mAb and rIL-2 was started on Day 10; test or control mAb (K8 antiidiotype) was injected i.p. on Days 10, 13, and 17 (1 mg/injection in 1 ml of PBS/BSA); rIL-2 was injected i.p. (5×10^4 units in 0.5 ml of PBS/BSA) 3 times daily on Days 10 to 14 and 17, or s.c. [2×10^5 units in 0.2 ml of incomplete Freund's adjuvant, 3% (w/v) BSA (40)] on Days 10, 17, and 24. Control treatment consisted of K8 mAb or rIL-2 alone or rIL-2 in combination with K8 mAb. Highly purified human rIL-2 produced in *Escherichia coli* (41) was a generous gift from Eurocetus, Amsterdam. The growth of the s.c. tumors was monitored once a week as described above.

Antibody-dependent Cellular Cytotoxicity. Short-term ^{51}Cr release experiments and long-term [^{3}H]thymidine release experiments were performed essentially as described (42, 43). In brief, mouse effector cells, *i.e.*, spleen cells or PEC, were mixed with 10^3 ^{51}Cr-labeled target cells (labeling with ^{51}Cr: 6.4 mBq/10^6 cells incubated at 37°C for 60 to 120 min; specific activity of ^{51}Cr, 13 to 22 GBq/mg of chromium; Amersham, Buckinghamshire, England) or 5×10^3 [^{3}H]thymidine-labeled target cells (labeling with [^{3}H]thymidine: cells incubated for 18 to 20 h with 3 mBq of [^{3}H]thymidine/ml; specific activity of [^{3}H]-thymidine, 247.9 GBq/mmol; Amersham) at E/T ratios varying from 100:1 to 6.25:1 in the 96-well round-bottomed microtiter plates (Costar). Test or control mAbs were added, at indicated concentrations, to a final volume of 200 μl. Subsequently the plates were centrifuged for 5 min at 1000 rpm and incubated for 4 h (^{51}Cr-labeled target cells) or 24 to 48 h ([^{3}H]thymidine-labeled target cells) at 37°C in humidified air with 5% CO_2. After this incubation period, the plates were centrifuged again (5 min, 1000 rpm), and the 100-μl supernatant was harvested and processed for determining the ^{51}Cr or [^{3}H]thymidine content in a GAMMA-8000 gamma counter or an LS8000 liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA).

The percentage of specific label release was calculated according to

the following formula

$$\% \text{ of specific label release} = (T - S/M - S) \times 100\%$$

where T is cpm in test sample, M is maximal releasable label in 2% (v/v) Triton X-100/0.5% (w/v) SDS/1% (w/v) sodium deoxycholate/10 mM EDTA, and S is spontaneously released label from target cells alone. The spontaneous label release never exceeded 15% of maximally releasable radioactivity.

Proliferation Inhibition Assay. PEC of BALB/c mice were harvested as described above. The cells were seeded in 96-well flat-bottomed microtiter plates (Costar). After 24-h incubation at 37°C in humidified air with 5% CO_2, nonadhering cells were removed by washing the wells once with medium. Test or control mAbs, at the indicated concentrations, as well as Daudi target cells (5000 target cells/well, E/T ratios varied from 5:1 to 0.6:1) were added to a final volume of 150 μl. Three days later, 50 μl of [^{3}H]thymidine (4.8 kBq/well; specific activity 247.9 GBq/mmol; Amersham) were added for the last 4 h of culture. Subsequently, the cells were harvested with a Titertek cell harvester (Flow Lab, Inc., McLean, VA), and the incorporated [^{3}H]thymidine was determined in the LS8000 liquid scintillation counter (Beckman). The percentage of specific inhibition of [^{3}H]thymidine incorporation (% of specific inhibition) was calculated according to the following formula

$$\% \text{ of specific inhibition} = (C - T/C - S) \times 100$$

where C is cpm incorporated in targets incubated with PEC and control mAbs, T is cpm incorporated in target cells incubated with PEC and test mAbs, and S is cpm incorporated in PEC alone.

RESULTS

Development of a Xenotransplantation Model of Human B-Cell Lines Transplanted in Athymic Nude Mice. Because it is well documented that it is very difficult to successfully transplant human primary leukemia/lymphoma cells in nude mice (44–46), established human B-cell lines were used for developing a xenotransplantation model. Nilsson and coworkers (46) described human Burkitt's lymphoma cell lines as the fastest growing human tumors in nude mice. Therefore, Burkitt's lymphoma cell lines BJAB, Daudi, EB3, RAMOS, Jiyoye, and Namalwa were used in the initial xenotransplantation experiments. These cell lines, however, failed to grow when injected i.p. or s.c. (5×10^6 cells) in C3H or C57BL/6 Kh nude mice. In BALB/c nude mice of less than 8 wk, the Daudi cells eventually grew when they were injected s.c. Sublethal whole-body irradiation (3 Gy) of BALB/c nude mice decreased the tumor latency time and reduced the variability of the growth rate of Daudi cells injected s.c. in these nude mice. In subsequent experiments, the growth kinetics of different doses of Daudi cells, injected s.c. in 3-Gy-irradiated BALB/c nudes, was studied (Fig. 1). From these data, it is clear that a s.c. injection of 5 to 20 × 10^6 Daudi cells invariably gave rise to the development of s.c. tumor nodules and that the growth rate of the tumor was relatively independent of the number of cells injected. These results guided our decision to inject 10 × 10^6 Daudi cells s.c. in 3-Gy-irradiated BALB/c nude mice for immunotherapy experiments.

Immunotherapy with Isotype Variants of CLB-CD19 mAb. Immunotherapy experiments were performed in the xenotransplantation model described above. On Day 0, the mice received 3 Gy of whole-body irradiation prior to the s.c. injection of 10 × 10^6 Daudi tumor cells. mAbs were injected i.p. on Days 0, 3, and 6 [1 mg/injection, in 1 ml of 0.5% PBS (w/v)/BSA]. Control animals received 1 ml of PBS (Experiment 1) or 1 mg of irrelevant mAb (K8, IgG2a isotype in 1 ml of PBS/BSA; Experiments 2 and 3) injected i.p. on Days 0, 3, and 6.

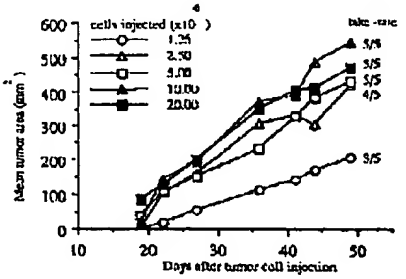

Fig. 1. On Day 0, the mice were irradiated (3 Gy) prior to the s.c. injection of Daudi tumor cells (5 mice/group). Indicated is the mean tumor area of the mice that did develop a tumor. At the end of each *curve*, the take-rate is indicated. The standard deviation of these measurements was approximately 20%.

Table 1 *Immunotherapy with IgG1, IgG2b, and IgG2a isotype variants of CLB-CD19 mAb*

On Day 0, the mice were irradiated (3 Gy) prior to the s.c. injection of 10 × 10^6 Daudi cells. Depicted is the number of mice with tumor/total number of mice (take rate) on Day 60. Statistical analysis was performed with the Fisher exact test.

	Take rate (Day 60)				
Treatment[a]	Experiment 1	Experiment 2	Experiment 3	Total	*P*
PBS/control mAb[b]	5/5	3/5	4/4	12/14	
CLB-CD19 IgG1	5/5	5/5	5/5	15/15	NS[c]
CLB-CD19 IgG2b	5/5	ND	ND	5/5	NS
CLB CD19 IgG2a	1/4	2/5	2/5	5/14	≤0.02

[a] mAbs were given i.p. (1 mg in 1 ml of PBS/BSA) on Days 0, 3, and 6.
[b] Control treatment consisted of 1 ml of PBS/BSA (Experiment 1) or 1 mg of K8 mAb (Experiments 2 and 3), injected i.p. on Days 0, 3, and 6.
[c] NS, not significant; ND, not done.

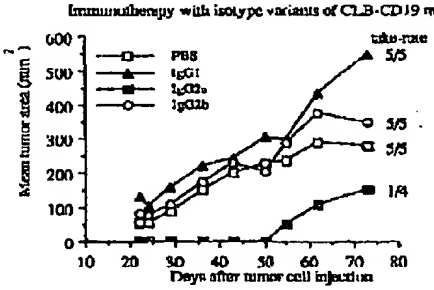

Fig. 2. On Day 0, the mice were irradiated (3 Gy) prior to the s.c. injection of 10 × 10^6 Daudi cells. Therapy was started 30 min after the tumor cell injection (1 mg of mAb i.p. in 1 ml of PBS/BSA) and was repeated on Days 3 and 6. Control animals received 1 ml of PBS/BSA injected i.p. on Days 0, 3, and 6. The standard deviation of these measurements was approximately 20%.

The results of three such experiments are shown in Table 1. In Fig. 2, the result of Experiment 1 is shown in terms of the growth rate of the tumor nodules, and the take-rate is indicated at the end of each curve. The results show that treatment of the mice with CLB-CD19 IgG2a mAb resulted in a reduction of the tumor take-rate ($P < 0.02$; Table 1) and an extension of the tumor latency time (Fig. 2), while treatment with the two other isotype variants of CLB-CD19 mAb had no significant effect on these parameters.

Possible Mechanism of the IgG2a CLB-CD19 mAb-mediated *in Vivo* Antitumor Effect. Because differences in plasma half-life ($t_{1/2}$) of the isotype variants of CLB-CD19 mAb could explain their different antitumor effect *in vivo*, the $t_{1/2}$ of the isotype variants of CLB-CD19 mAb was determined. BALB/c mice were injected i.p. with 1 mg of mAb, and at various time intervals, the plasma CLB-CD19 mAb level was determined by

indirect immunofluorescence, in which mean fluorescence intensity obtained with the plasma samples was interpolated on a standard curve of intensities obtained with CLB-CD19 mAb solutions of known concentrations. The $t_{\frac{1}{2}}$ (days ± SD, $n = 6$) of the IgG1, IgG2b, and IgG2a was 7.4 ± 2.5, 6.8 ± 1.9, and 6.8 ± 1.4, respectively. This result indicates that it is not likely that the different therapeutic activities of the isotype variants are caused by differences in distribution and/or breakdown *in vivo*.

To identify the involved effector mechanisms, *in vitro* ADCC experiments were performed. In these ADCC experiments, using mouse spleen cells or PEC as effector cells, none of the CLB-CD19 mAb isotype variants was able to induce $CD19^+$ target cell lysis, while target cells sensitized with the anti-HLA Class II mAb R24.3 were readily lysed by PEC (data not shown) and, although to a limited extent, also by freshly isolated BALB/c nude mouse spleen cells as shown in Fig. 4.

CLB-CD19 mAb-mediated effects were obtained with a modification of a cytostasis assay (11). In this assay, Daudi target cells are incubated for 3 to 4 days with mouse PEC in the presence of specific mAbs or irrelevant isotype-matched control mAbs. At the end of this incubation period, the target cell survival is quantitated by measuring [^{3}H]thymidine incorporation. The results of such a representative experiment are shown in Fig. 3. The R24.3 mAb, rat IgG2b directed against human HLA Class II antigens, was found to be very potent in this assay. Of the IgG1 and IgG2a isotype variants of CLB-CD19 mAb included in this experiment, only the latter was able to inhibit significantly the [^{3}H]thymidine incorporation of the target cells. In an identical experiment, the IgG2b isotype variant was equally ineffective as was the IgG1 isotype variant shown in Fig. 3 (data not shown). The results obtained with these experiments correlate with the isotype-dependent antitumor activity of CLB-CD19 mAb observed *in vivo* and therefore, suggest that the effector cells in PEC that are responsible for the antiproliferative effect *in vitro* may also be responsible for the *in vivo* antitumor effect of the treatment with mAb alone.

Combination Therapy with IgG2a CLB-CD19 mAb and rIL-2. When IgG2a CLB-CD19 mAb therapy was delayed until Day 10, instead of started immediately after tumor cell injection (1 mg of mAb-i.p. on Days 10, 13, and 17), the antitumor effect was not significantly different from control-treated animals (Table 2). However, when this delayed mAb treatment was combined with rIL-2, the antitumor effect was again significant (Table 2). Treatment with rIL-2 alone had no effect on the growth of Daudi tumor cells. This result indicates that rIL-2 is able to potentiate the therapeutic efficacy of IgG2a CLB-CD19 mAb *in vivo*.

ADCC Activity of rIL-2-activated BALB/c Nude Mouse Spleen Cells. As already mentioned before, freshly isolated, nonactivated BALB/c nude mouse spleen cells were poor ADCC effector cells; only with the R24.3 mAb could some specific ^{51}Cr release be obtained. However, activation of these spleen cells in rIL-2 resulted in a dramatic increase in ADCC activity with all tested mAbs as shown in Fig. 4. This result suggests that rIL-2-induced ADCC activity of lymphoid effector cells may contribute to the observed potentiation by rIL-2 of IgG2a CLB-CD19 mAb-mediated antitumor activity *in vivo*.

DISCUSSION

In this paper we present evidence that mAbs directed against the B-cell-specific antigen CD19 powerfully inhibit the growth of human Burkitt's Daudi lymphoma cell line transplanted into nude mice. Of IgG1, IgG2b, and IgG2a isotype switch variants of CLB-CD19 mAb, only the IgG2a isotype exhibited this antitumor activity. Furthermore, we show for the first time in a xenotransplantation model that rIL-2 dramatically potentiates the therapeutic effect of tumor-specific mAbs.

The observed superior antitumor activity of the IgG2a isotype variant is in agreement with previously published studies by other investigators (14, 15). However, in these studies, IgG2b and, to an even lesser extent, also IgG1 showed some antitumor activity. In our study these isotypes were completely inactive.

Most investigators ascribe the mechanism of *in vivo* antitumor activity, mediated by tumor-specific antibodies, to ADCC reactions (13–15, 42). Therefore, we performed extensive *in vitro* ADCC assays with mouse spleen cells or mouse PEC as effector cells. However, none of the CLB-CD19 isotype variants was able to mediate ADCC activity with these effector cells. A modification of a cytostasis assay (11), called the proliferation inhibition assay, eventually gave positive results; PEC inhibited the proliferation of Daudi target cells *in vitro* only in the presence of the IgG2a isotype variant of CLB-CD19 mAb and

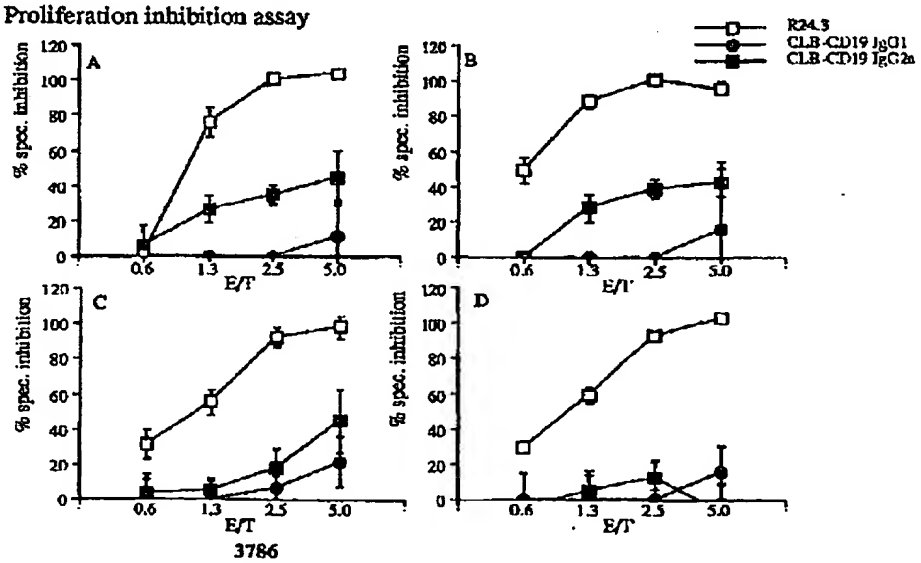

Fig. 3. PECs were seeded in 96-well flat-bottomed microtiter plates. After 18 to 24 h, Daudi target cells were added (5000 cells/well) and different concentrations of test or control mAb: *A*, R24.3 culture supernatant, dilution of 1/3, 7.5 μg/ml of CLB-CD19 mAb; *B*, R24.3 culture supernatant, dilution of 1/12, 2.5 μg/ml of CLB-CD19 mAb; *C*, R24.3 culture supernatant, dilution of 1/24, 0.8 μg/ml of CLB/CD19 mAb; and *D*, R24.3 culture supernatant, dilution of 1/48, 0.3 μg/ml of CLB/CD19 mAb. R24.3 mAb, rat IgG2b directed against human HLA Class II antigens, was included as a positive control. Indicated is the percentage of specific inhibition ± SD ($n = 3$), calculated according to the formula described in "Materials and Methods."

Table 2 *Combination therapy with IgG2a CLB-CD19 mAb and rIL-2*

On Day 0, the mice were irradiated (3 Gy) prior to the s.c. injection of 10×10^6 Daudi cells. Depicted is the number of mice with tumor/total number of mice (take-rate) on Day 60. Statistical analysis was performed with the Fisher exact test.

Treatment[a]	Take rate				
	Experiment 1	Experiment 2	Experiment 3	Total	*P*
Control mAb	3/5	4/4	8/9	15/18	
Control mAb + rIL-2[b]	ND[c]	ND	6/8	6/8	NS
rIL-2[d]	5/5	3/4	ND	8/9	NS
CLB-CD19 IgG2a	2/5	4/5	6/9	12/19	NS
CLB-CD19 IgG2a + rIL-2[d]	0/4	1/3	3/6	4/13	≤0.01
CLB-CD19 IgG2a + rIL-2[b]	ND	ND	2/8	2/8	≤0.02

[a] mAbs were given i.p. (1 mg in 1 ml of PBS/BSA) on Days 10, 13, and 17.
[b] rIL-2 was given s.c. (2×10^5 units in 0.3 ml of Freund's incomplete adjuvant, 3% BSA) on Days 10, 17, and 24.
[c] ND, not done; NS, not significant.
[d] rIL-2 was given i.p. (5×10^4 units in 0.5 ml of PBS/BSA) 3 times daily on Days 10, 11, 12, 13, 14, and 17.

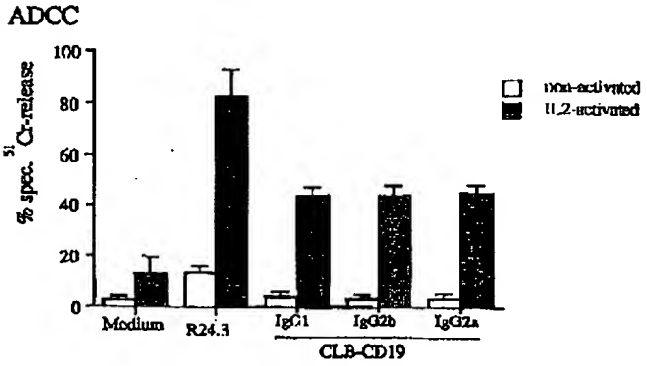

Fig. 4. Spleen cells from BALB/c nude mice were used as effector cells in a 4-h ^{51}Cr release experiment. Before these effector cells were used, dead cells and erythrocytes were removed by centrifugation over Ficoll-Hypaque. Nonactivated (freshly isolated) or IL-2-activated (6 days in 500 units of rIL-2/ml) cells were mixed with 10^4 ^{51}Cr-labeled target cells at an E/T ratio of 50:1 in the absence of mAb (medium) or in the presence of R24.3 mAb (1 μg/ml) or the isotype variants of CLB-CD19 mAb (2.5 μg/ml). Indicated is the mean percentage of specific ^{51}Cr release ± SD of triplicate determinations.

not in the presence of the other two isotype variants (Fig. 3). Because this proliferation inhibition assay measures the net result of antibody-dependent cellular effector functions including ADCC, phagocytosis, and/or cytostasis of antibody-coated tumor cells, the exact mechanism and the identity of the effector cell in the PEC population remain to be elucidated. The effector cells most likely are macrophages because the assay is performed with adherent PECs, which consist of over 90% macrophages. However, we cannot exclude the possibility that, in the adherent PEC population, a considerable fraction of the cells are Thy-1-positive T-cells.

In contrast to the results of the experiments shown in Table 1, the antitumor activity of mAb alone treatment was lost when this treatment was delayed until Day 10 after tumor cell inoculation (Table 2). An obvious explanation for this observation is that, by Day 10, the tumor burden is too great to be eradicated by antibody-dependent effector mechanisms. While treatment with rIL-2 alone did not affect the growth of the Daudi cells in the nude mice, the combination of IgG2a CLB-CD19 mAb with rIL-2, started on Day 10, resulted in a strong antitumor effect (Table 2). From these results we conclude that rIL-2 increased the therapeutic efficacy of IgG2a CLB-CD19 mAbs in the nude mice. This result is in agreement with previously published studies in syngeneic animal models (23, 28–30).

As already mentioned, fresh nude mouse spleen cells did not mediate ADCC activity irrespective of the isotype of the CLB-CD19 mAb used to sensitize the CD19$^+$ target cells. However, culture of these spleen cells *in vitro* with rIL-2 induced ADCC activity with all isotypes of CLB-CD19 mAb. In view of this result we are currently testing whether rIL-2 addition to the treatment with IgG1 or IgG2b CLB-CD19 mAb would result in antitumor activity in our xenotransplantation model. Our results clearly show that IL-2 can enhance the ADCC activity of BALB/c nude mouse spleen cells. This is in contrast with the results published by Berinstein *et al.* (23). In their study the culture of spleen cells in 2500 units of rIL-2/ml for 3 days did not induce ADCC activity. A possible explanation for this discrepancy could be that we used spleen cells from nude mice, which are enriched for NK cells (47), while Berinstein *et al.* used spleen cells from immunocompetent mice. The results of our ADCC experiments suggest that rIL-2-induced ADCC activity of lymphoid effector cells may contribute to the *in vivo* observed increased antitumor activity of mAb and rIL-2 combination therapy. Since it is known that IL-2 activation increases the expression of various surface molecules, such as IL-2 receptor (CD25), HLA-DR, transferrin receptor, and leu 23, on cultured human NK cells (48–52), a possible mechanism of the enhancement of ADCC by IL-2 could be an increased expression and/or function of FcR on ADCC effector cells. Moreover, it was shown recently that FcR (CD16)-ligand interaction on human NK cells increased the expression of CD25 and the production of the lymphokines tumor necrosis factor and interferon-γ (53), suggesting a linked regulation of the activation of FcR and IL-2 receptor expression.

The rat mAb R24.3, which is directed against HLA Class II antigens, proved to be very potent in the proliferation inhibition assay (Fig. 3) and in ADCC assays with rIL-2-stimulated nude mouse spleen cells (Fig. 4). R24.3 mAb was identified as rat IgG2b isotype and, indeed, this rat isotype is known to be very efficient in activating complement and in mediating ADCC reactions with human and mouse effector cells (18). However, the results obtained in these *in vitro* assays with R24.3 mAb cannot directly be compared with the results obtained with CLB-CD19 mAb, because it is possible that HLA Class II and CD19 antigen densities and the affinity of the mAbs for their antigens are very different. We therefore are in the process to produce rat mAbs, preferentially of the IgG2b isotype, directed against CD19 in order to be able to compare the antitumor activity of such a reagent *in vivo* and *in vitro* with the mouse CLB-CD19 mAb.

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